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Extracellular sphingosine-1-phosphate: a novel actor in human glioblastoma stem cell survival properties

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Ai miei genitori

con immenso affetto e gratitudine.

CONTENTS

ABBREVIATIONS

ABC	ATP-binding cassette
bFGF	Basic fibroblast growth factor
Cer	Ceramide
DMEM	Dulbecco's Modified Eagle's Medium
DHSph	Dihydrosphingosine
EGF	Epidermal growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GlcCer	Glucosylceramide
GSCs	Glioblastoma stem cells
LacCer	Lactosylceramide
MGMT	O^6-methyl-guanine DNA methyl-transferase
SK	Sphingosine kinase
SKI	Sphingosine kinase inhibitor
SM	Sphingomyelin
Sph	Sphingosine
S1P	Sphingosine-1-phosphate
TMZ	Temozolomide

SUMMARY

Glioblastoma multiforme (GBM) is the most frequent and aggressive intracranial tumour in humans. The prognosis of GBM patients remains unfavourable even after aggressive treatments based on multiple approaches, due to the high proliferation rate, migrating-invasive properties, and resistance to therapeutic intervention. The introduction of the alkylating agent TMZ in glioblastoma therapy has improved patient survival, but drug resistance mechanisms limit its benefits.

The aim of this study was to provide a contribution to the understanding of the malignant and chemoresistance properties in GBM by focusing on the role of the bioactive sphingoid molecules ceramide and S1P, which act as antagonists in regulating cell properties and survival.

Accumulating literature indicates that ceramide is a tumour suppressor sphingolipid, able to induce antiproliferative and apoptotic responses, and that it is able to act as a major player in the mechanism of action of many chemotherapeutic drugs. We demonstrated that the treatment of T98G human glioblastoma cells with cytotoxic TMZ concentrations results in a significant increase in intracellular ceramide, which in turn promotes cell death. On the other hand, TMZ is not able to induce ceramide accumulation in TMZ-resistant glioblastoma cells (TMZ-R). These data suggest a role of ceramide as a mediator of TMZ-induced toxicity.

A large amount of evidence underlines the role of S1P as an important tumour-promoting sphingolipid, acting predominantly in the extracellular milieu after interaction with specific G protein-coupled receptors and exerting opposite effects on cell survival compared to ceramide. Parallel studies demonstrated that S1P secretion in TMZ-R cells is functional to inhibit the cytotoxic effect of ceramide and to confer TMZ-resistant properties to glioblastoma cells.

Stimulated by these findings, we next evaluated the role of sphingolipid mediators in the malignant features of glioblastoma stem cells (GSCs), a cell subpopulation within the tumour mass involved in the aberrant expansion and therapy resistance properties of glioblastomas. To this purpose we used GSCs isolated from the human U87-MG glioblastoma cell line and GSCs isolated from a primary culture of human glioblastoma. We found that both GSC models efficiently form typical neurosphere structures in mitogen-defined medium and express high levels of recognized cancer stem cell markers. Moreover, GSCs exhibit resistance to TMZ at concentrations that are cytotoxic in U87-MG, despite not expressing the DNA repair protein MGMT, a major contributor to TMZ-resistance. Even though a large amount of evidence underlines that S1P is able to

favor growth, invasion and chemotherapy resistance of glioblastoma cells, so far little is known on the possible role of S1P as a factor modulating GSCs malignant properties.

Further experiments revealed that glioblastoma cells and GSCs are able to efficiently synthesize S1P and also to release it in the culture medium. Notably the intracellular S1P level was found much lower in GSC models than in the glioblastoma cell line; meanwhile the extracellular S1P level was significantly higher in GSC models than in U87-MG cells. These differences resulted in an extracellular S1P-intracellular S1P ratio at least 10 times higher in GSCs compared to U87-MG. Furthermore, this ratio is about 1:1 in both GSCs, thus suggesting that these cells are an efficient source of S1P in the extracellular microenvironment. Furthermore we found that ceramide-extracellular S1P ratio is at least 2-fold lower in GSCs than in U87-MG. Since S1P and ceramide exert opposing effects on cell survival, according to the "sphingolipid rheostat" model, this different ratio could promote GSC survival observed after TMZ treatment.

Interestingly, enzyme activity assays excluded the presence of sphingosine kinase (SK), the enzyme responsible for S1P byosinthesis, in GSC medium, implicating an efficient secretion of S1P in GSCs.

The analyses of the expression of the ABC-transporters known to be involved in S1P export (ABCG2, ABCA1 and ABCC1), revealed that only ABCA1 is expressed in GSCs. Notwithstanding, after ABCA1 inhibition, no variations in S1P release was observed, suggesting that other mechanisms different from those known are involved.

We also investigated the role of S1P in glioblastoma resistance to TMZ. A first interesting finding was that exogenously administered S1P protected U87-MG cells against TMZ cytotoxic effects. In addition, we found that, after co-treatment with TMZ and an inhibitor of S1P biosynthesis, GSCs became sensitive to the toxic effect of the drug. Of note, exogenous S1P administration was able to revert this effect. These data strongly support extracellular S1P as an important mediator in TMZ-resistance of GSCs.

Furthermore, results obtained in GSCs isolated from two patients affected by glioblastoma with different aggressive phenotype, revealed that the extracellular release of S1P was significantly higher by cells isolated from the most aggressive tumour, suggesting that the release and thus the levels of extracellular S1P might be related to tumour aggressiveness and patient prognosis.

In conclusion, our data implicate for the first time GSCs as an important source of S1P in the extracellular microenvironment, where, on its turn, S1P can act as an autocrine/paracrine messenger able to contribute to the GSC survival properties. A better understanding of S1P role in GSCs aggressive phenotype could represent a critical start point that sets the bases for the development of new compounds able to sensitize GSCs to chemotherapeutic treatments, thus improving survival rates in GBM patients.

INTRODUCTION

1. HUMAN GLIOMAS

1.1 Glioma features

Gliomas are the most common primary brain tumours in humans and an important cause of mental impairment and death [1]. As central nervous system tumours, they are characterized by special features that differentiate them from all the other tumours. First, the distinction between benign and malignant lesions is less apparent than in other locations. Some glial tumours with benign histological features, such as low mitotic index, cell uniformity and slow growth, can infiltrate entire regions of the brain, showing a clinically malignant behaviour. In addition, the anatomical location of the tumour can have fatal consequences regardless of histological features, for instance an expanding benign tumour can lead to a condition of brain compression resulting in severe neurological impairment. Furthermore, even the most malignant gliomas rarely metastasize outside of the central nervous system [2].

Gliomas include a group of highly heterogeneous cancers classified, according to cell line derivation and differentiation morphological evidence, in: astrocytoma, oligodendroglioma, oligoastrocytoma, ependymoma and choroid plexus tumours [3]. Astrocytomas are the highest incidence gliomas (75% of all gliomas) [4], sorted by the World Health Organization (WHO) in different classes, distinguished by the presence of specific histological characteristics such as nuclear atypia, mitotic activity, endothelial proliferation and necrosis [5]. In particular, it is possible to distinguish four different classes of astrocytomas: pilocytic astrocytoma (grade I), diffuse or fibrillary astrocytoma (grade II), anaplastic astrocytoma (Grade III), and glioblastoma multiforme (grade IV). The tumours belonging to the class I and II are considered with low-grade malignancy, while those of III and IV class are considered to be high-grade malignant [5]. Glioblastoma multiforme or simply glioblastoma (GBM) (WHO grade IV) is the most frequent malignant primary brain tumour and it is one of the most aggressive cancer. Indeed, GBM is characterized by one of the worst survival rates of all the human cancers with a median survival ranging from nine to twelve months [1,3]. Patient survival does not improve even after aggressive treatment with multiple approaches, such as surgery, chemotherapy and radiotherapy. The surgical approach often cannot be applied without functional loss of large areas of central nervous tissue with severe neurological impairment [6].

As defined by the term “multiforme”, GBM comprises morphologically heterogeneous neoplasms, in which the cellular composition can be highly variable. GBM is characterized by uncontrolled cell proliferation, diffuse infiltration, massive angiogenesis, high genomic instability, strong resistance to apoptosis and then to radio-chemotherapy, all distinguishing features representing useful prognostic factors for overall survival [7-9].

Two classes of GBM have been identified: primary GBM (or GBM de novo) and secondary GMB [3]. Primary GBM accounts for about 90% of GBM cases and it is diagnosed without evidence of a previous lower-grade tumour. Primary GBM generally occurs in elderly patients (60-70 years old) and it is characterized by a very short clinical history (less than 3 months). On the other hand, secondary GBM, representing the minority of GBM cases, derives from a lower-grade tumour that undergoes the process of tumour progression to higher malignancy grades. Secondary GBM usually occurs in adults between fifty and sixty years, and the time of progression from lower to higher degree of malignancy ranges from months to decades [9,10].

1.2 Altered signalling pathways in glioblastoma tumorigenesis

Despite glioblastomas are strikingly heterogeneous, common alterations in specific cellular signal transduction pathways and cellular functions occur in most malignant gliomas. These alterations include pathways involved in the regulation of important cellular processes, such as proliferation, survival, differentiation, migration, DNA repair and apoptosis. In particular, the main pathways altered in glioblastomas include p53, pRB, growth factors, PI3K/Akt, apoptosis and angiogenesis signalling (Fig. 1) [3,10-13].

p53 pathway

The p53 protein is one of the most important tumour suppressors. Its expression is up-regulated in response to conditions of cellular stress, such as γ and UV radiation, hypoxia, DNA damage and inappropriate oncogene activation. p53 acts as a transcription factor promoting the expression of genes that block the cell cycle and genes that repair DNA. If the amount of damage is beyond the cell’s capacity for repair, p53 favours the expression of genes involved in apoptosis, promoting cell death.

p53 inactivating mutations are frequent in secondary GBM. Alterations of regulators of p53 levels and/or activity are common in primary GBM. For example, Mdm2 protein is a negative regulator of p53: Mdm2 binds to p53 promoting its ubiquitination and subsequent degradation via proteasome. Mdm2 is overexpressed in some GBM. Furthermore, the $p14^{ARF}$ protein, which negatively regulates the Mdm2 ability to bind p53, is

often deleted in malignant gliomas or the corresponding gene is methylated so that its expression is inhibited. The abnormal p53 pathway functioning leads to a greater tolerance to DNA damages resulting in genomic instability [11,13].

pRb pathway

The retinoblastoma protein (pRb) is a tumour suppressor involved in the regulation of the cell cycle, controlling cells transition from the G1 into the S phase. Cyclins/cyclin-dependent kinase (Cdk) complexes and Cdk inhibitors play a fundamental role in the regulation of cell proliferation. In particular, the cyclin D/Cdk4 complex catalyses the pRB phosphorylation resulting in the release of E2F transcription factors sequestered by the pRB binding, which are essential for the expression of the S-phase-genes, thus inducing DNA synthesis. Moreover, the $p16^{INK4a}$ protein inhibits cell cycle progression, negatively regulating the formation of the complex cyclin D/Cdk4. Inactivating mutations of pRB and $p16^{INK4a}$ or the amplification of genes coding for Cdk4 and cyclin D are typical features of glioblastomas. All of these changes cause the pRB inability to bind E2F, resulting in an uncontrolled cell cycle progression from G1 to S phase [11,13].

Growth factor signalling

Different growth factors can inappropriately activate proliferation in most malignant gliomas. The main growth factors involved in determining the GBM phenotype are: epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF1) and the vascular endothelial growth factor (VEGF). Tumours activate the corresponding pathway through the following mechanisms: ligand or receptor over-expression, receptor mutation causing its constitutive activation in the absence of the ligand, activation of intracellular messengers through mutation and/or a change of expression levels and the loss of negative regulators. The activated growth factor pathways regulate numerous pro-tumorigenic cell functions able to improve proliferation, resistance to apoptosis, motility, invasion, and neo-angiogenesis [9,10].

PI3K /Akt/PTEN pathway

Among the pathways altered in glioblastomas there is also the phosphatidylinositol 3-kinase (PI3K)/Akt (PKB)/PTEN (Phosphatase and tensin homolog) signalling. PI3K are a family of kinases that phosphorylate membrane lipids, in particular phosphoinositides and phosphatidylinositols, such as the phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5) P_2) which is converted in phosphatidylinositol 3,4,5-triphosphate (PtdIns (3,4,5) P_3). PI3K activation can occur through integrin signalling, receptors with seven transmembrane

domains coupled to G proteins or growth factors tyrosine-kinases receptor. The PtdIns (3,4,5) P_3 binds to different cytosolic proteins, including Akt which undergoes a conformational change that facilitates its activation through phosphorylation in correspondence of the two amino acid residues T308 and S473 by phosphoinositide-dependent kinases (PDK) 1 and 2, respectively.

PTEN is a phosphatase that negatively regulates the PI3K/Akt pathway, by converting PtdIns (3,4,5) P_3 in PtdIns (4,5) P_2, thus directly antagonizing PI3K activity. Akt is a key regulator of cell proliferation and survival pathways. It is a Serine/Threonine kinases able to inhibit apoptosis, promote cell proliferation and regulate lipids and glucose metabolisms, cell movements and vesicle trafficking.

High levels of phosphorylated/activated Akt and deletions or mutations in the PTEN gene are the most common alterations of the PI3K/Akt signalling in glioblastomas. These changes are crucial in determining the GBM malignant features, such as rapid tumour growth, invasiveness, resistance to cytotoxic treatments and massive angiogenesis [3,12,14].

Apoptosis

A hallmark feature of malignant glioma cells is an intense resistance to death-inducing stimuli such as radiotherapy and chemotherapy. This biological property has been linked to genetic alterations of key regulatory molecules involved in mitogenic signalling, most prominently growth factors and the PI3K/Akt/PTEN signalling axis, as well as regulatory and effector molecules residing in classical cell death networks of both extrinsic (death receptor-mediated) and intrinsic (mitochondria-dependent) apoptosis signalling pathways [15,16].

The “death receptors” are cell surface molecules that, upon binding their ligands, recruit adapter molecules to provide a molecular scaffold for the autoproteolytic processing and activation of caspases. The most important death receptor systems include TNFR1, TRAILR1-2 and CD95. These death receptors are often down-regulated and their ligand mutated in glioma pathogenesis [12].

The role of the anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-XL, MCL-1, CED-9) in gliomagenesis has also been extensively studied. These proteins modulate apoptosis signalling by preserving mitochondrial membrane integrity and the release of cytochrome c, thus blocking the caspase cascade and the apoptotic program. These anti-apoptotic Bcl-2 family members are often up-regulated by overexpression in glioblastomas [12].

Angiogenesis

GBMs are among the most highly vascular of all solid tumours. Microvascular hyperplasia, the defining histopathological phenotype of both primary and secondary GBM, consists of proliferating endothelial cells that emerge from normal parent microvessels accompanied by stromal elements, including pericytes and basal lamina. Angiogenesis is vital for macroscopic solid tumour growth and one common feature in the transition from low-grade to secondary GBM is a dramatic increase in microvascular proliferation. In particular, in glioma pathogenesis frequent alterations of different genetic programs converging on a final common angiogenesis, include positive (VEGF, PDGF, bFGF, IL-8, SDF-1) and negative (thrombospondin1, thrombospondin2, endostatin, tumstatin, interferons) regulators of this process [12].

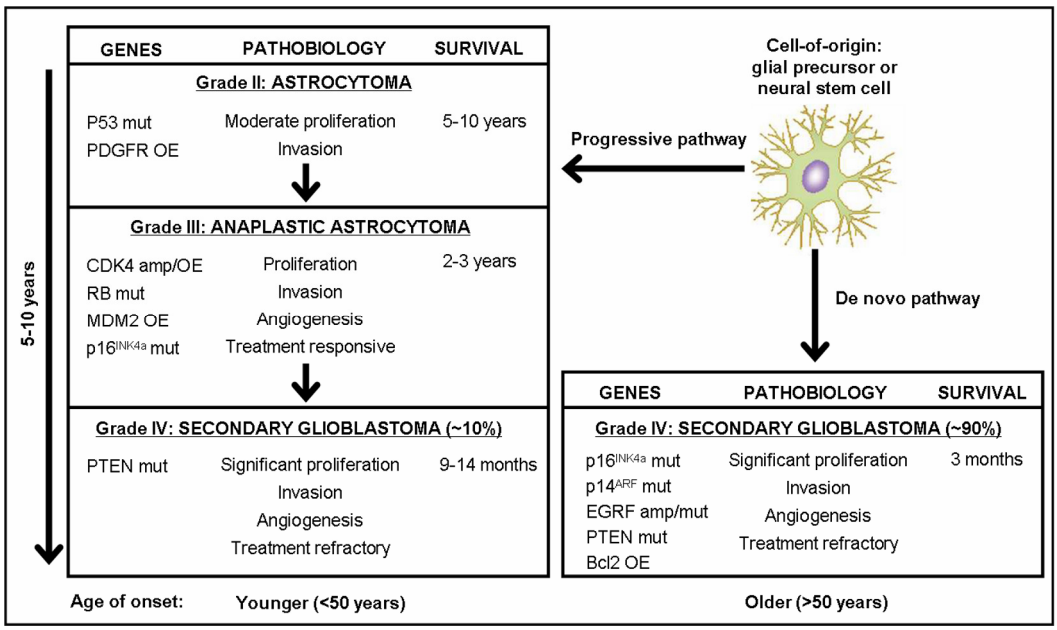

Figure 1. Altered signalling pathways in glioblastoma tumorigenesis (modified image from Furnari et al. [12]).

The relationships between survival, pathobiology and the molecular lesions that lead to the formation of primary (de novo) and secondary (progressive) glioblastomas are shown. OE, overexpressed; amp, amplified; mut, mutated.

1.3 Glioblastoma treatment

Glioblastomas are among the most devastating tumours and most difficult cancers to treat. GBM grow very quickly and show a high resistance to apoptosis, thus to radio-chemotherapy [1,3]. Furthermore, brain tumour therapy is even more complex for drug distribution within the intracranial space, due to the presence of the blood-brain barrier. The standard therapy for malignant gliomas involves surgical resection when feasible, radiotherapy, and chemotherapy. However, very poor prognosis is still associated with GBM, being the median survival of patients approximately 1 year [1].

Malignant gliomas cannot be completely eliminated surgically because of their infiltrative nature, but patients should undergo maximal surgical resection whenever possible. Surgical debulking reduces the symptoms from mass effect and provides tissue for histological diagnosis and molecular studies. Advances such as MRI (magnetic resonance imaging)-guided neuronavigation, intraoperative MRI, functional MRI, intraoperative mapping [17] and fluorescence-guided surgery [18] have improved the safety of surgery and increased the extent of resection that can be achieved.

Radiotherapy is the mainstay of treatment for malignant gliomas. The addition of radiotherapy to surgery increases survival among patients with glioblastomas from a range of 3 to 4 months to a range of 7 to 12 months [19]. It has been reported that chemotherapeutic agents may enhance the effectiveness of radiotherapy, resulting in a modest increase in survival (a 6 to 10% increase in the 1-year survival rate) [20,21]. In the last years, Stupp and colleagues demonstrated that the combination of radiotherapy and temozolomide (TMZ), an oral alkylating agent with good penetration of the blood–brain barrier, followed by adjuvant temozolomide therapy, had an acceptable side-effect profile and, as compared with radiotherapy alone, increased the median survival (14.6 months vs 12.1 months, $p<0.001$) [22]. In addition, the survival rate at 2 years among the patients who received radiotherapy and temozolomide was significantly greater than the rate among the patients who received radiotherapy alone (26.5% vs 10.4%), establishing radiotherapy with concomitant and adjuvant temozolomide as a useful combination for newly diagnosed glioblastomas.

1.4 Temozolomide in glioblastoma treatment

As described above, in recent years TMZ has become the current mainstay of anti-glioblastoma therapy, ameliorating patient prognosis. TMZ is also used in clinical treatment of metastatic melanoma and has shown promising effects in refractory acute leukemia [23-25]. TMZ is an alkylating agent fully active when administrated orally (100% bioavailability). This compound has excellent pharmacokinetic properties and it is able to cross the blood brain barrier due to its lipophilic character, causing minimal side effects [26-28].

TMZ is an imidazotetrazin and, after intestinal adsorption, at physiologic pH and without hepatic activation, it is spontaneously hydrolyzed to its active metabolite MTIC (methyl-triazen-imidazole-carboxamide). MTIC is an unstable compound, which undergoes degradation with the generation of the inactive 5-aminoimidazole-4-carboxamide (AIC), which spontaneously produces molecular nitrogen, and the eletrophilic methyldiazonium cation. The methyl-diazonium is a highly reactive molecule acting as an alkylating agent, resulting in the methyl group transfer on the DNA (Fig. 2) [29]. In particular alkylating agents are able to methylate nucleophilic sites (mainly groups containing nitrogen, oxygen and sulphur) of cellular macromolecules. These agents could potentially damage all cellular macromolecules; however, their biological effects, i.e. cytotoxicity and mutagenicity, are due to their ability to damage DNA. The alkylating agents bind covalently to DNA nucleophilic residues, establishing intermolecular and intramolecular cross-linking. The oxygen and nitrogen atoms present in the DNA bases are the main target of alkylation. The main damaged DNA sites are the N^7 or O^6 positions of guanine residues (accounting for nearly 70% and 7% of the methylated lesions, respectively) and N^3 position of adenine residues (10% of lesions). However, although it is the minor product of alkylation, the methylation of the O^6 position of guanine is considered the major mediator of temozolomide toxicity. Guanine alkylation leads to changes in the DNA structure and functions:

- alteration of the genetic code, because the structure of the alkylated guanine favours the formation of a base pair with the abnormal insertion of a thymidine instead of a cytosine during subsequent DNA replication (base/base mismatches);
- cleavage of the guanine imidazole ring;
- loss of a guanine residue and consequent breaking of the DNA helix [30].

Summarizing, the therapeutic benefit of temozolomide depends on its ability to alkylate/methylate DNA. This methylation damages the DNA and triggers tumour cells death.

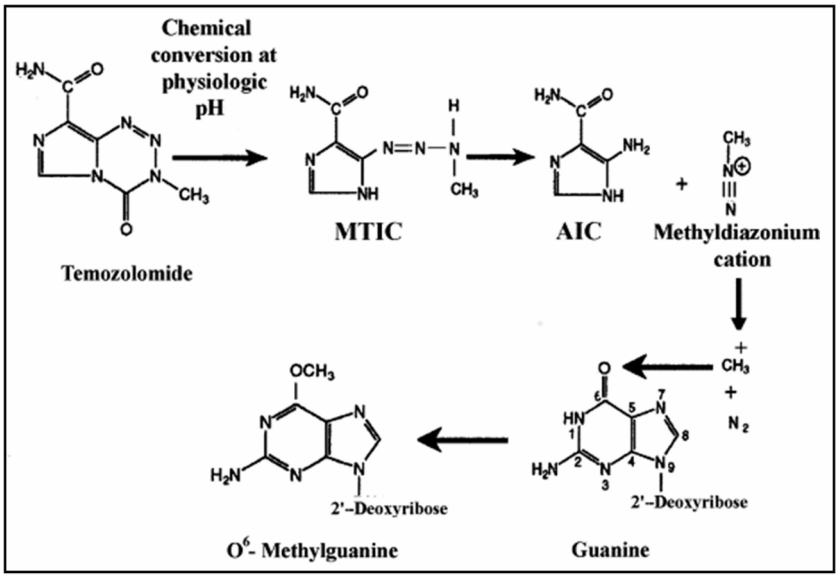

Figure 2. Activation and mechanism of action of TMZ [30].

Although the mechanism of cell death induced by the therapeutic treatment with TMZ is not yet entirely clear, extensive evidence shows that this drug induces the processes of apoptosis or autophagy.

Sur and co-workers [31] demonstrated that TMZ is able to induce apoptosis in T98G cells from human glioma. This evidence has been confirmed through morphological techniques (TUNEL assay) which demonstrated the presence of nuclear DNA fragmentation after TMZ treatment. Apoptosis induction occurs through the intrinsic activation pathway with the down-regulation of the pro-apoptotic Bax levels and the up-regulation of the anti-apoptotic Bcl-2, these events are related to the activation of cysteine proteases such as calpain and caspase-3.

On the other hand, Kanazawa and colleagues [32] demonstrated that the treatment of T98G human glioma cell line with TMZ induces the activation of the autophagy process. This evidence has been demonstrated by a morphological assay based on the use of acridine orange which allows detecting the formation of acidic vesicular organelles, typical of the autophagy process. Moreover, a biochemical analysis showed an increased expression of the LC3 protein (microtubule-associated protein 1-ligth chain 3), marker of the autophagic process, after TMZ treatment.

1.5 Temozolomide resistance

Despite the introduction of the alkylating agent temozolomide in glioblastoma therapy has improved patient survival, drug resistance mechanisms limit its benefits and the prognosis remains unfavorable [33]. The biological effects of TMZ and cell resistance to them depend on at least three DNA repair systems, (a) O^6-alkylguanine-DNA-alkyltransferase, called also methyl-guanine methyl-transferase (MGMT); (b) mismatch repair (MMR) and (c) base excision repair (BER) (Fig. 3) [34,35].

One of the best characterized mechanisms of TMZ resistance is the protein MGMT. MGMT is a small enzyme-like protein that removes small alkyl adducts from the O^6 position of the DNA guanine through a stoichiometric and auto-inactivating reaction. This reaction consists in a covalent transfer of the alkyl group from the alkylated sites in DNA to a conserved cysteine residue (Cys 145) within the active site of the MGMT protein. This transfer causes the irreversible inactivation of MGMT which is subsequently ubiquitinated and undergoes degradation via proteasome. This is the reason why MGMT is considered a “suicide enzyme“ [30]. MGMT is ubiquitously expressed in normal human tissues and its expression is often higher in malignant tissues that in the normal counterpart. MGMT appears to be overexpressed in malignant gliomas and represents the major obstacle in the treatment of these cancers, because it repairs the DNA damages caused by TMZ, resulting in poor patient survival [36]. MGMT expression can be induced by glucocorticoids, cyclic AMP, and protein kinase C (PKC) activators. Furthermore, MGMT expression is also regulated by the methylation status of specific CpG regions of its gene-promoter. Hypermethylation of these MGMT promoter regions (found in about 45% of gliomas) silences the gene, resulting in loss of expression and is strongly associated with prolonged survival in malignant glioma patients treated with temozolomide [37].

Since high levels of MGMT are responsible for resistance to TMZ, its inhibition represents a possible target to improve drug sensitivity. In fact, it has been demonstrated that the treatment with MGMT inhibitors - i.e. O^6-benzylguanine or O^6-(4-bromotenyl)guanine (Lomeguatrib) - sensitizes human glioma cells to TMZ treatment. This evidence suggests that a therapy combination with temozolomide and MGMT inhibitors may be an effective tool to treat resistant gliomas [38].

MMR is represented by a protein complex dedicated to the repair of biosynthetic errors generated during DNA replication. The MMR system recognizes base mismatches and insertion-deletion loops, cuts the nucleotide sequence containing the lesion, and restores the correct base sequence. Therefore, not only MGMT but also MMR is involved in target cell susceptibility to TMZ.

However, the MMR system does not suppress, but instead promotes the cytotoxic effects of TMZ. In fact, MMR is not able to repair the incorrect base pairing determined by treatment with TMZ, failing to find a

complementary base for the methylated guanine. According to a predominant hypothesis, this event causes reiterated "futile" attempts of damage repair, with the generation of long-lived nicks in the DNA, leading to the activation of cell cycle arrest and apoptosis [30,39]. A deficiency in the MMR pathway, resulting from mutations in any one or more of the proteins forming the complex repairing DNA, has been observed in malignant gliomas. This deficiency lead to tolerance of TMZ-generated DNA adducts, a continuation of DNA replication, and a loss of the cytotoxic effects of temozolomide [40,41].

BER removes DNA lesions due to physical or chemical agents. In particular, BER is able to repair N^7-methylguanine and N^3-methyladenine determined by treatment with alkylating agents, such as TMZ. Recent attempts to overcome resistance to temozolomide conferred by mismatch repair deficiency have focused on blocking base excision repair. One of the strategies used to block base excision repair includes the inhibition of a key enzyme, the poly(ADP-ribose) polymerase 1, PARP1 [42], which binds to and is activated by DNA strand breaks, is thought to be important in protecting and trimming the DNA ends for repair synthesis [43]. In presence of PARP1 inhibitors, the repair process of N-methylpurines cannot be completed and the deriving DNA strand breaks contribute to cytotoxicity. Several studies demonstrated that the inhibition of PARP1 increases the efficacy of temozolomide in the treatment of malignant glioma cells and this effect was especially evident in tumours deficient in DNA mismatch repair, since cancer cells are tolerant to O^6-methylguanine damage and show low sensitivity to TMZ [44,45]. These studies indicate a role of the repair of N-methylpurine adducts in the resistance to the antitumour activity of temozolomide and other alkylating agents. Thus, a pharmacological strategy based on the interruption of N-methylpurine repair might represent a novel strategy to restore or increase glioma sensitivity to TMZ.

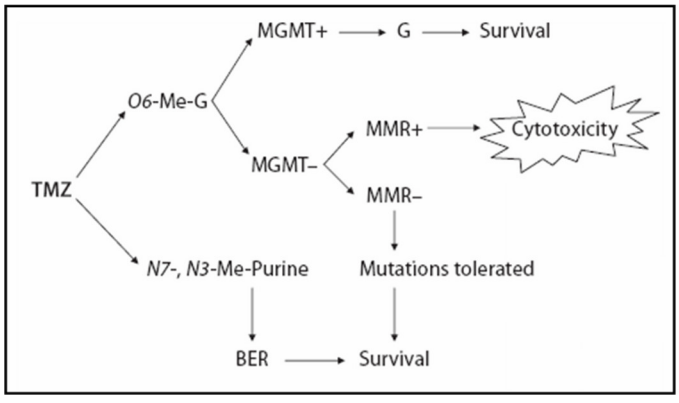

Figure 3. Summary of TMZ resistance mechanisms (modified image from Zhang et al. [35]).

1.6 Glioblastoma stem cells

For more than a century, human cancers have been recognized as a morphologically heterogeneous population of cells. What has become clear in the past 10 years is that these cells are also functionally heterogeneous [46]. Recent studies suggest the existence of a subpopulation of cells within the tumour mass (about 3-5% of the total cancer cell population), named cancer stem cells (CSCs) which play a crucial role in both the initiation and maintenance of cancer, and might play an important role in its malignant behaviour. This model of cancer growth is called hierarchical model (or cancer stem cell hypothesis). This postulate implies that the bulk of cancer cells within a tumour are progeny of CSCs, have no tumorigenic potential, thus cannot regenerate new tumours, and might represent a mix of partially differentiated cancer progenitor-like cells with limited proliferative capacity, terminally differentiated, an death committed cancer cells [46].

CSCs have now been identified and isolated in different tumours: haematopoietic, breast, prostate, colon, head and neck and pancreas cancers [46]. Moreover, the presence of cancer stem cells has been recently demonstrated in central nervous system tumours, particularly in GBM [47-51].

Glioblastoma stem cells (GSCs), similarly to the normal neural stem cell counterpart (NSCs), are able to self-renew, thus able to go through numerous cycles of cell division while maintaining their undifferentiated state. Moreover, GSCs emerged as multipotent, thus able to simultaneously differentiate into multiple lineages (neuron, astrocytes and oligodendrocytes). More importantly, these cells were described as tumour-founding cells, able to regenerate a phenocopy of the original GBM when injected in immunocompromised mice. Notably, GSCs are also highly resistant to chemo-radiotherapy [50-53]. These characteristics suggest that, despite GSCs represent a minority component of the tumour mass, they are critical in determining the GBM initiation, progression and malignancy.

Another peculiarity of GSCs is the increased expression of the main brain cancer stem cell markers: the cytoskeleton protein nestin and the membrane glycoprotein CD133 [54-56]. Nestin is an intermediate filament (IF) protein expressed in the stem/progenitor proliferating cells during the central nervous system developmental stages and its expression is down-regulated in differentiated cells. It may be involved in the organization of the cytoskeleton, cell signalling and metabolism, organogenesis, and represents the proliferation, migration and multi-differentiated characteristics of multi-lineage progenitor cells [56]. CD133 (also named as Prominin) is a membrane glycoprotein expressed in normal human neural stem cells, and it is down-regulated in differentiated cells. Five alternative promoters, three of which are partially regulated by methylation, drive the transcription of several mRNA isoforms of CD133. Its localization is in membrane protrusions, which suggests an involvement in the mechanisms influencing cell polarity, migration and

interaction of stem cells with neighbouring cells and/or extracellular matrix [54]. Nestin and CD133 expression may be a potential indicator of the biological aggressiveness of gliomas. These 2 proteins can be considered as markers of tumour burden, and recurrence in human gliomas [55].

However these molecules are not the universal enriched markers for GSCs. Indeed it has been observed that the stage-specific embryonic antigen CD15 could be also a good candidate to isolate GSCs. CD15+ cells are often also CD133+ and, within the tumour, are able to self-renewal, show a multilineage differentiation potential and are highly tumorigenic *in vivo* [57].

The GSC origin remains a subject of ongoing debate in the actual scientific literature. Current hypothesis postulate that GSCs either originate from transformed neural stem cells or neural progenitor in the brain or that they de-differentiate from mature brain mutant cells and reacquire phenotypic and functional characteristics of neural stem cells (Fig. 4) [52,56].

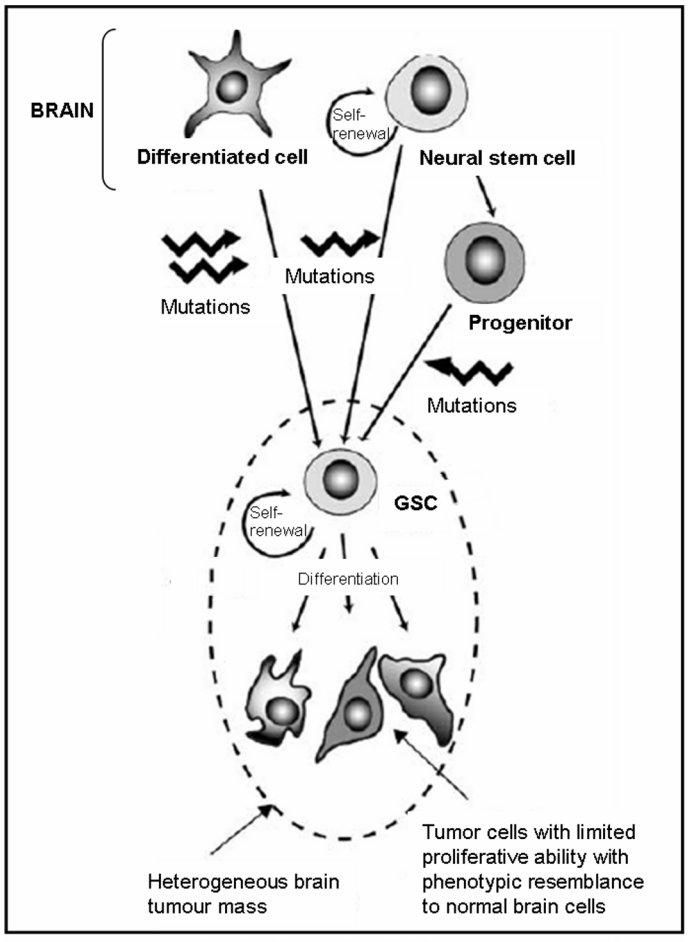

Figure 4. GSC origin (modified image from Singh et al. [47]).

1.7 Altered molecular pathways in glioblastoma stem cells

The GSC component differs from normal neural stem cells for a complete de-regulation of the major signal transduction pathways involved in fundamental cellular processes such as cell proliferation, differentiation, stemness maintenance, as well as drug- and radio- resistance [58]. The main pathways altered in GSCs include Notch, Hedgehog-GLIs, growth factors, BMP, and TGF-β signalling (Fig. 5).

Notch pathway

Notch proteins are a family of single transmembrane domain receptors involved in cell-cell communication. Upon binding with one of its ligands (Jagged 1-2, Delta like 1-3-4), Notch is cleaved by the γ-secretase complex. The Notch intracellular domain is thus released from the plasma membrane, and translocates into the nucleus where it acts as a transcription factor. In the nervous system, Notch is able to induce proliferation and self-renewal of neural stem cells while suppressing their differentiation. Alterations of Notch signalling are frequent in GSCs. In particular, Notch overexpression and its ligand-independent activation were observed. The de-regulation of this signalling pathway causes an increased GSC growth and proliferation [59,60].

Hedgehog pathway

The binding of the Hedgehog ligands to their receptors activates transducers termed GLIs (named for their discovery in gliomas), which then translocate into the nucleus to activate or repress downstream targets. The Hedgehog pathway is one of the key regulators of embryogenesis and is critical for the survival of several different types of normal stem cells, including neural stem cells [61]. Hedgehog signalling is also active in gliomas and contributes to GSCs function, favouring cell proliferation, self-renewal, tumorigenicity and drug resistance [62]. Furthermore, it has been demonstrated that Hedgehog pharmacologic inhibitors (i.e. cyclopamine) improve traditional therapy efficiency against gliomas. In particular, Bar and colleagues demonstrated that cyclopamine treatment improves the effects of radiation on GSC survival [63].

Growth factors signalling

GSCs are also characterized by the deregulation of growth factor signalling (EGF, bFGF, PDGF and IGF). GSCs activate these pathways through different mechanisms, such as ligand and/or receptor overexpression, receptor mutation causing its constitutive activation, intracellular messenger activation through mutation or the loss of expression of negative regulators [59].

The signal initiated by the binding between growth factors and their receptors (Receptor Tyrosine Kinases, RTKs) is transduced and amplified through downstream molecule cascades, such as the pro-survival AKT/PI3K pathway. Upon activation, AKT promotes survival, proliferation, invasion, and secretion of pro-angiogenic factors. It has been recently demonstrated that GSCs are more dependent on AKT signals than matched non-stem glioma cells. Pharmacologic inhibitors of AKT attenuate the generation of neurospheres, the structures usually formed by GSCs in culture, suggesting that AKT inhibition may specifically target the GSC population to reduce tumour malignancy [64].

BMP pathway

Bone Morphogenic Proteins (BMPs) are a family of growth factors named for their central roles in bone and cartilage formation. Most BMPs elicit their actions through binding to cell-surface receptor kinases (the BMPRs). This binding activates, through phosphorylation, Smad1/5/8 proteins that bind to the co-activator Smad4, translocate into the nucleus, and regulate transcription. BMPs are crucial factors that regulate proliferation and apoptosis in neural stem cells and usually promote the differentiation of these cells [58]. Lee and collaborators demonstrated that this pathway is altered in GSCs. In particular, in GSCs the BMP receptor expression is regulated by epigenetic alterations leading to a reduced expression and signal transduction. In this way GSCs are able to escape the differentiation process induced by BMPs, thus keeping intact their stemness [65].

TGF-β pathway

The TGF-β superfamily includes a large number of proteins capable of regulating crucial cellular processes such as differentiation and cellular development [59]. Recent experimental evidence demonstrated that GSCs are able to produce and release TGF-β into the extracellular microenvironment, which acts in an autocrine/paracrine way inducing cell survival and stemness maintenance through the activation of Sox2 signal transduction pathway [66].

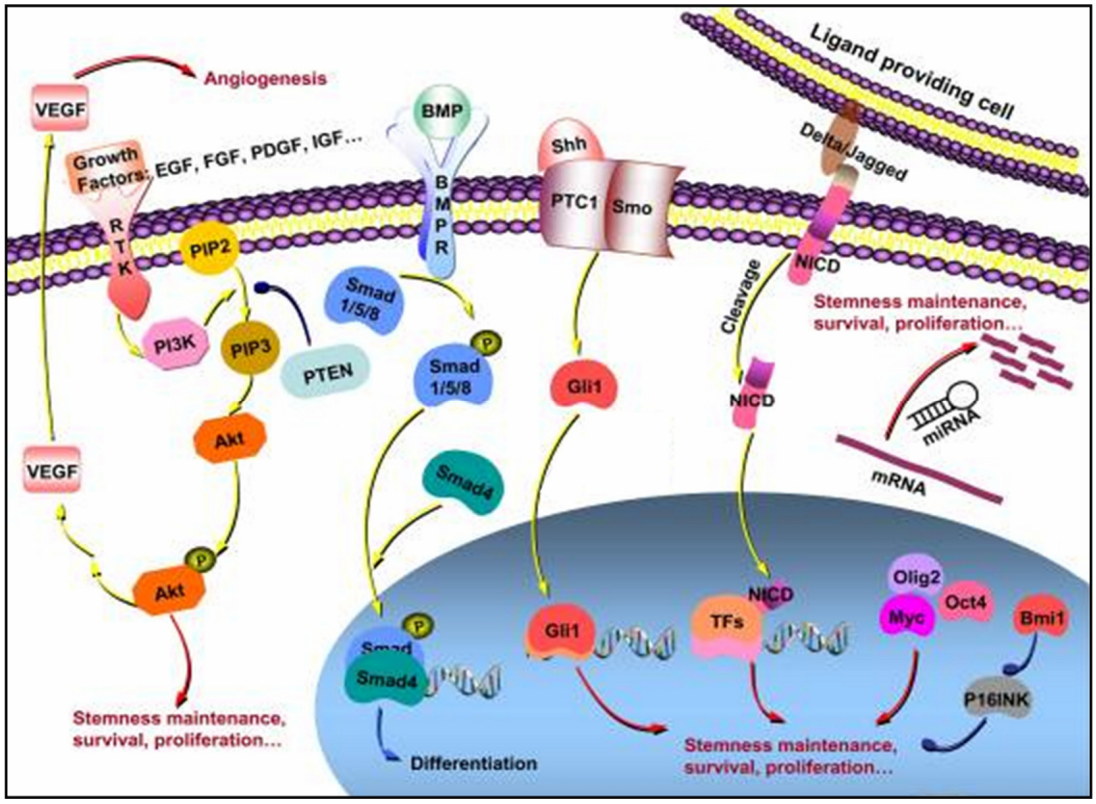

Figure 5. Complex signalling pathways and cellular factors regulate GSCs [58].

Transcription factors, epigenetic regulators, and miRNAs are extremely powerful regulators of normal and cancer cells. They are capable of simultaneous regulation of multiple downstream targets and are implicated also in the maintenance of GSC properties. Many evidence shows that the main factors involved are: Sox2, Oct4, Nanog, Olig 2, c-Myc and Bmi1 [58].

Sox2-Oct4-Nanog

Sox2, Oct4 and Nanog are transcription factors crucial for the regulation of the balance between self-renewal and differentiation in embryonic and adult stem cells [59]. Oct4 is highly expressed in many human glioma specimens and cell lines, and its expression correlates with the glioma grade [67]. The direct role of Oct4, Sox2 and Nanog in glioma CSCs is not well understood, but overexpression of Oct4 in rat C6 glioma cells increases the expression level of the stemness marker Nestin. These data suggest that Oct4 may inhibit the differentiation of glioma CSCs and contribute to CSC stemness maintenance [67].

Olig2

Olig2 is a transcription factor that is almost exclusively expressed in the central nervous system. During brain development, Olig2 is expressed in neural progenitor cells that give rise to oligodendrocytes and certain neuronal subtypes [58]. Functionally, Olig2 is expressed in both normal neural stem cells and glioma CSCs. Olig2 sustains the replication-competent state of neural progenitors and is necessary for the multilineage differentiation potential of neural progenitors. In GSCs Olig2 regulates the process of cell proliferation and inhibits the differentiation process through the suppression of the cell cycle regulatory protein $p21^{WAF1/CIP1}$ [68].

c-Myc

c-Myc is a transcription factor, considered as an oncoprotein, extensively studied for its instrumental role in the proliferation of both normal stem cells and tumour cells. It has been recently demonstrated that glioma CSCs express high levels of c-Myc and that c-Myc is required both for maintenance of GSCs *in vitro* and for their tumorigenic capacity *in vivo* [69]. It has been demonstrated that c-Myc additionally prevents differentiation and promotes self-renewal of GSCs derived from a p53/PTEN double knock-out mouse model [70].

BMI1

BMI1 belongs to the Polycomb group genes, which usually function as epigenetic silencers. BMI1 has been implicated in determining stem cell fate in multiple tissues and is a positive regulator of neural stem cell self-renewal. BMI1 is also a known oncogene frequently overexpressed in many cancer types, including gliomas [58]. As demonstrated by Abdouh and colleagues, BMI1 is overexpressed also in GSCs and is required to sustain the self-renewal process of these cells [71].

miRNAs

miRNAs are small noncoding RNAs that can silence target genes through post-transcriptional mechanisms on target mRNAs. miRNAs are powerful intracellular regulators because a single miRNA can regulate many distinct mRNAs. In cancer biology, miRNAs can function as oncogenes or as tumour suppressors.

Two recent reports directly investigated the roles of miRNAs in glioma CSCs. The levels of miR-124 and miR-137 are reduced in grade III and IV malignant gliomas in comparison with normal brain. Overexpression of these two miRNAs inhibits proliferation while inducing differentiation of glioma CSCs, indicating a tumour

suppressor role for these two miRNAs in GSCs [72]. Similarly, another miRNA, miR-451, is expressed at lower levels in $CD133^+$ GSCs in comparison with $CD133^-$ non-stem glioma cells. In particular, it has been demonstrated that miR-451 inhibits the growth of glioma CSCs and disrupts the formation neurosphere, the structures usually formed by GSCs in culture [73].

1.8 Glioblastoma stem cells in tumour pathogenesis

Despite advances in treatment strategies that combine surgery with radiotherapy and chemotherapy, GBM remains one of the most deadly disease with a high rate of recurrence after treatment. Growing evidence suggests that GSCs play a crucial role in the GBM malignant behaviour, being involved in the processes of radio and chemo-resistance, recurrence, metastasis and angiogenesis [53,56,74].

Radiotherapy is one of the most important therapeutic approaches in GBM treatment, but in most cases it becomes a simple palliative treatment due to the presence of a population of radio-resistant tumour cells. Gliomas usually respond to radiation treatment, but subsequently radiation resistant cells recur. GSCs appear to contribute to the onset of radio-resistance. In fact, Bao and colleagues demonstrated that GSCs, in response to DNA damage induced by ionizing radiation or by radio mimic drugs, rapidly activate the response to DNA damage by phosphorylation of proteins fundamental in this mechanism such as ATM, Rad17, Chk1 and Chk2. The activation of these proteins allows the arrest of the cell cycle and a rapid repair of the DNA damage, thus favouring cell survival and consequently the onset of radio resistance. In particular, CD133-expressing tumour cells preferentially activate the DNA damage check points in response to radiation, and repair radiation-induced DNA damage more effectively than CD133-negative tumour cells [75]. Chalmers proposed that up-regulated and hyper-responsive cell cycle checkpoint pathways in GSCs may be a potential target for therapy [76].

A novel approach for GBM treatment is the pharmacological treatment by the administration of the alkylating agent TMZ concurrently during radiotherapy, followed by adjuvant TMZ therapy. As described above, this drug has a cytotoxic effect on tumour cells, as it induces the methylation of guanine at the O^6 position, causing the formation of DNA adducts favouring the cell cycle arrest and the activation of the cell death process.

It has been demonstrated that GSCs are resistant to cytotoxic drugs. This intrinsic property is frequently related to the high expression levels of the DNA repair protein MGMT, which is capable of removing the methyl groups added to the DNA by alkylating agents. This characteristic makes GSCs insensitive to

pharmacological treatment, thus increasing the tumour survival [77]. Growing evidence suggests that also active drug extrusion mechanisms could play a crucial role in drug resistance observed in GSCs. In particular, it has been demonstrated that GSCs are characterized by an increased expression of the ABCG2 transporter, member of the ABC transporter family [77,78]. Also ABCB1 (or PgP), and multi-drug resistance proteins 3 (MDR-3) were found overexpressed in GSCs [74]. The high expression of these proteins able to transport outside the cells anticancer drugs such as Temozolomide, Etoposide, Paclitaxel and Carboplatin, is related to the ability of these cells to escape the pharmacological treatment with the onset of drug resistance. Moreover, also alteration in the apoptotic and autophagic machinery could contribute to GSC chemoresistance. The down-regulation of the main autophagy-related proteins, such as Beclin1, ATG5 and LC3-II has been observed in GSCs. The expression levels of these proteins are low, even after TMZ treatment [79]. GSCs are also characterized by an overexpression of apoptosis suppressor such as Bcl2, Bcl-XL, FLIP and several inhibitors of apoptosis, such as IAPs proteins which bind and inhibit caspases 3-7-9, preventing apoptosis [77,80].

Malignant gliomas are characterized by florid angiogenesis, with neovascularization significantly correlated with enhanced tumour aggressiveness, degree of tumour malignancy, and poor clinical prognosis. Indeed, active angiogenesis plays a key role in providing oxygen and nutrients to the tumour mass as well as facilitating metastasis. Given the importance of CSCs in glioma maintenance, it is not surprising that GSCs and angiogenesis are tightly connected. Bao and colleagues demonstrated that, in comparison with matched non-stem cancer cells, GSCs have a stronger capacity for promoting angiogenesis, partially through amplified secretion of VEGF, one of the most important pro-angiogenic factors [81].Treating GSCs with the VEGF-neutralizing antibody Bevacizumab attenuates their ability to promote angiogenesis both *in vitro* and *in vivo*. The mechanism underlying the specific up-regulation of VEGF in GSCs is still unclear, but it has been suggested that environmental factors such as hypoxia and acidosis play important roles in this process [58].

Notwithstanding, the precise mechanisms underlying the chemoresistance and the malignancy of GSCs are not completely understood. Thus, the molecular characterization of GSCs represents a critical step in defining glioblastoma properties, and may be essential in developing effective therapeutic strategies for the complete eradication of the tumour.

2. SPHINGOLIPIDS

Sphingolipids are essential constituents of eukaryotic cell membranes. For long time they have been considered merely as structural components, fundamental in the definition of the membrane bilayers. In the last 20 years advances in biochemical and molecular research led to the identification of sphingolipids acting as intra- and extra-cellular messengers involved in the regulation of crucial aspects of cell biology such as cell growth, death, migration, senescence and inflammatory response [82].

Sphingolipids are amphipathic molecules, characterized by a hydrophilic (or polar head) and a hydrophobic (or tail) portion. The structural element common to all sphingolipids is represented by ceramide (Cer). It consists of a long-chain sphingoid base, linked via amide bond to a long-chain fatty acid, predominantly palmitic (C_{16}) or stearic acid (C_{18}) (Fig. 6). Sphingosine (Sph), an amino-alcohol containing 18-20 carbon atoms (C-18 or C-20) and characterized by the presence of a double bond across C4-C5, is the most frequent sphingoid base constituting Cer in humans, followed, in very small proportions, by C-18/C-20 sphinganine (which lacks the double bond C4-C5). Both are in the *trans* D-*erythro* form.

Cer is the central building block of all sphingolipids, and represents the hydrophobic portion, or tail, of these molecules. Through its primary alcoholic residue, Cer can be conjugated to different hydrophilic groups, which represent the polar head of sphingolipids. Cer binding to phosphocholine or saccharidic structures leads to the generation of sphingomyelins (SM) and glycosphingolipids (GSLs), respectively. The oligosaccharide portion of sphingolipids may contain 15-20 saccharidic units, among which, the most frequent are glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and different species of sialic acid, linked to Cer through a β-glycosidic bond. Furthermore, both the sphingoid base (sphingosine) and Cer may exist in the phosphorylated form in correspondence with the carbon in position 1 (C-1), thus the primary alcoholic residue is functionalized [83].

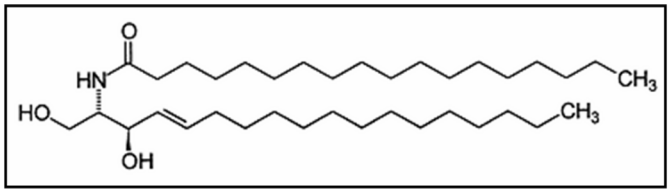

Figure 6. Ceramide chemical structure [84].

Sphingolipids are ubiquitous membrane components, being present in different organelle membranes and particularly abundant in the plasma membrane. Cer is present in small amounts within cell membranes, as it functions primarily as an intermediate of complex sphingolipid metabolism and acts as a cell signalling mediator [85]. In biological plasma membranes, SM and GSLs represent the major sphingolipids, displaying an asymmetric or polarized distribution, and play important roles in the regulation of membrane fluidity and sub-domain structures. In particular, GSLs are mainly localized in the outer leaflet of the cell membrane. Their saccharidic portion is exposed on the cell surface, while the hydrophobic portion, constituted by Cer, is inserted within the outer leaflet of the membrane. Glycosphingolipids show a distribution on the membrane specific for each cell type, which can vary with differentiation and neoplastic transformation [84].

Sphingolipids, thanks to their amphipathic properties, are able to diffuse laterally within the membrane and, together with cholesterol and specific proteins, may give rise to microdomains, specialized platforms able to compartmentalize cellular processes by serving as organizing centres for the assembly of signalling molecules [86].

2.1 Sphingolipid metabolism

Sphingolipids can originate from both the "de novo synthesis" or from the degradation of complex sphingolipids (Fig. 7) [87].

The "de novo" biosynthesis of sphingolipids begins with the condensation of palmitoyl-CoA with L-serine. This reaction is catalyzed by the enzyme serine-palmitoyl transferase (SPT) and leads to the formation of 3-ketosphinganine, which is subsequently reduced to sphinganine by a NADPH-dependent oxidoreductase, the 3- ketosphinganine reductase. Sphinganine is the substrate of the (dihydro)-Cer synthase (CerS), which uses an acyl donor (often palmitoyl-CoA, but also stearoyl-CoA) to bind sphinganine to a fatty acid, forming dihydroCer. In mammalian cells six different isoforms of CerS have been recently identified and are encoded by six different genes, members of the LASS family (Longevity Assurance Genes). Each of these genes specifically synthesise one of the several Cer species which differ in the fatty acid chain length. In particular, LASS1 is involved in the production of C_{18}Cer, LASS2 of C_{22}Cer, LASS3 of C_{18}/C_{24}Cer, LASS4 of C_{20}Cer, LASS5 and LASS6 of C_{16}Cer and C_{14}/C_{16}Cer respectively [88,89]. The product of the reaction catalyzed by CerS, dihydroCer, is desaturated in position 4,5 of the sphingoid base by a NADPH-dependent oxydoreductase, the dihydroCer desaturase (DES), with the consequent formation of Cer [90]. All the

enzymes involved in the de novo biosynthesis of Cer are localized in the endoplasmic reticulum (ER) membrane, acting on the cytosolic surface of the same, and the reaction products remain there anchored.

Cer is the common precursor for the synthesis of all complex sphingolipids. Through its hydroxyl group of the carbon in position 1, Cer can bind to additional functional groups, generating glycosphingolipids (GSL), Cer 1-phosphate and sphingomyelin (SM).

Glycosphingolipids derive from the conjugation of the Cer primary alcoholic residue with one or more saccharide units, through β-glycosidic bond.

Galactosylceramide (GalCer) is synthesized from Cer and UDP-galactose by the galactosyltransferase, enzyme expressed at the luminal side of the ER membrane in Schwann cells and oligodendrocytes. GalCer is an important lipidic component of myelin (formed by Schwann cells in the PNS and oligodendrocytes in the CNS), and confers rigidity and stability to the membranes, allowing a correct conduction of nerve impulses [91].

Glucosylceramide (GlcCer) is obtained from Cer and UDP-glucose, through a reaction catalyzed by GlcCer synthase (GCS), enzyme localized at the cytosolic leaflet of the Golgi apparatus [92]. So, GlcCer biosynthesis requires an efficient transport mechanism of Cer from the cytoplasmic side of the RE to the cytoplasmic side of the cis-Golgi. Once synthesized, GlcCer can be routed directly to the plasma membrane (via a vesicular system), or it can be further modified by subsequent glycosylations, with the consequent production of more complex glycosphingolipids, such as gangliosides. This process is catalyzed by different glycosyl-transferases acting by associating to GlcCer individual saccharide units, following a precise sequential order. The enzymes involved in these reactions are located and act in the luminal surface of the Golgi cisternae; a flippase enzyme mediating the translocation of GlcCer from the cytoplasmic to the luminal side of the cis-Golgi, so that subsequent glycosylations can take place [93,94].

Cer can also undergo phosphorylation of the hydroxyl group of the carbon in position 1 by the Cer kinase (CK), with the consequent formation of Cer 1-phosphate (Cer1P) [82]. The subcellular localization of this enzyme has not been definitely identified, but it seems to be at the level of the plasma membrane, the Golgi apparatus, and the cytoplasm [95].

SM represents approximately 10% of the lipids present in mammalian cells. SM synthesis is based on the transfer of phosphocholine from phosphatidylcholine to Cer, with the production of a diacylglycerol (DAG) molecule. This reaction is catalyzed by SM synthase (SMS). Several studies suggest the existence of 2 different enzymatic SMS isoforms: SMS1, localized in the luminal side of the cis/medial Golgi apparatus and SMS2, primarily localized to the plasma membrane [82,96]. Experimental evidence has shown that about

90% of the de novo synthesis of SM occurs in the cis/medial Golgi, and only a small percentage occurs at the level of the plasma membrane. So most of SM biosynthesis, as well as that of GlcCer, requires a mechanism of Cer transport from the RE, where it is synthesized, to the Golgi apparatus. The localization of SMS2 at the plasma membrane suggests its role as a regulator of SM and Cer levels for signalling pathways and signal transduction.

Sphingolipids reach their final destination at the plasma membrane mainly following the vesicular flow of exocytosis from the ER or the Golgi apparatus to the plasma membrane.

Membrane glycosphingolipids are constitutively degraded by a process involving endocytosis and the endo-lysosomal district; the enzymatic steps of this degradation process include exoglycohydrolases requiring an acidic pH, guaranteed within the lysosomal or endosomal vesicles, in order to perform their catalytic activity. These enzymes catalyze the glycosphingolipids degradation in monosaccharides and Cer components, determining the sequential hydrolytic detachment of monosaccharides [97].

SM degradation is catalyzed by sphingomyelinase (SMase), an enzyme able to hydrolyze the SM phosphodiester bond, with the consequent formation of Cer and phosphocholine. Three isoforms of this enzyme have been described and distinguished, according to their subcellular localization and the optimum pH, in acidic, neutral, and alkaline SMase. The acid SMase is located mainly in the lysosomal compartment. Another isoform of acidic SMase has been identified, due to a splice variant in the transcription of the encoding gene; this enzyme can be secreted or localized in the outer membrane leaflet [82,98]. The neutral SMase has various subcellular locations including the inner leaflet of the plasma membrane, ER, Golgi, and even the nucleus [98]. The localization of alkaline SMase is more restricted, being mainly expressed in the intestinal tract, and in the bile where it participates to SM digestion [99].

Cer is degraded by ceramidases (CDases); three isoforms of CDases have been identified and classified by their pH optima as acidic, neutral and alkaline CDases. These CDases are located at the plasma membrane, lysosome, and ER/Golgi complex, respectively [100]. These enzymes hydrolytically cleave Cer in fatty acid and sphingosine (Sph). Notably, Sph origin is exclusively catabolic, since it only derives from sphingolipids degradation [101].

Cer-derived Sph can be recycled or undergo phosphorylation in position C1 with the generation of sphingosine-1-phosphate (S1P) by sphingosine kinases (as described below). S1P can be metabolized through the irreversible cleavage in position C2-C3, to hexadecenal and phosphoethanolamine in a reaction catalyzed by the S1P lyase enzyme, located on the ER cytosolic side [102]. This reaction, and the SPT catalyzed one, are the reactions of the entire sphingolipid metabolism which occur in only one direction, the

enzymes catalysing the opposite reaction being absent. S1P can also be dephosphorylated back to Sph through a reaction catalyzed by either lipid phosphate, or S1P specific phosphatases [103-105].

The products obtained from the sphingolipids degradation such as saccharide residues, fatty acids and, particularly, sphingosine can be reused for the synthesis of complex sphingolipids. In this process of recycling, sphingosine produced from SM and GSL catabolism reaches the RE where it is N-acylated to Cer. Cer can then be used for the biosynthesis of complex sphingolipids, as described in *de novo* biosynthesis. This pathway is called "salvage pathway" [82]. The sphingosine recycling process is an energy advantage to cells and, in different cell types, such as cells of glial origin, may constitute the principal pathway of sphingolipids synthesis [106].

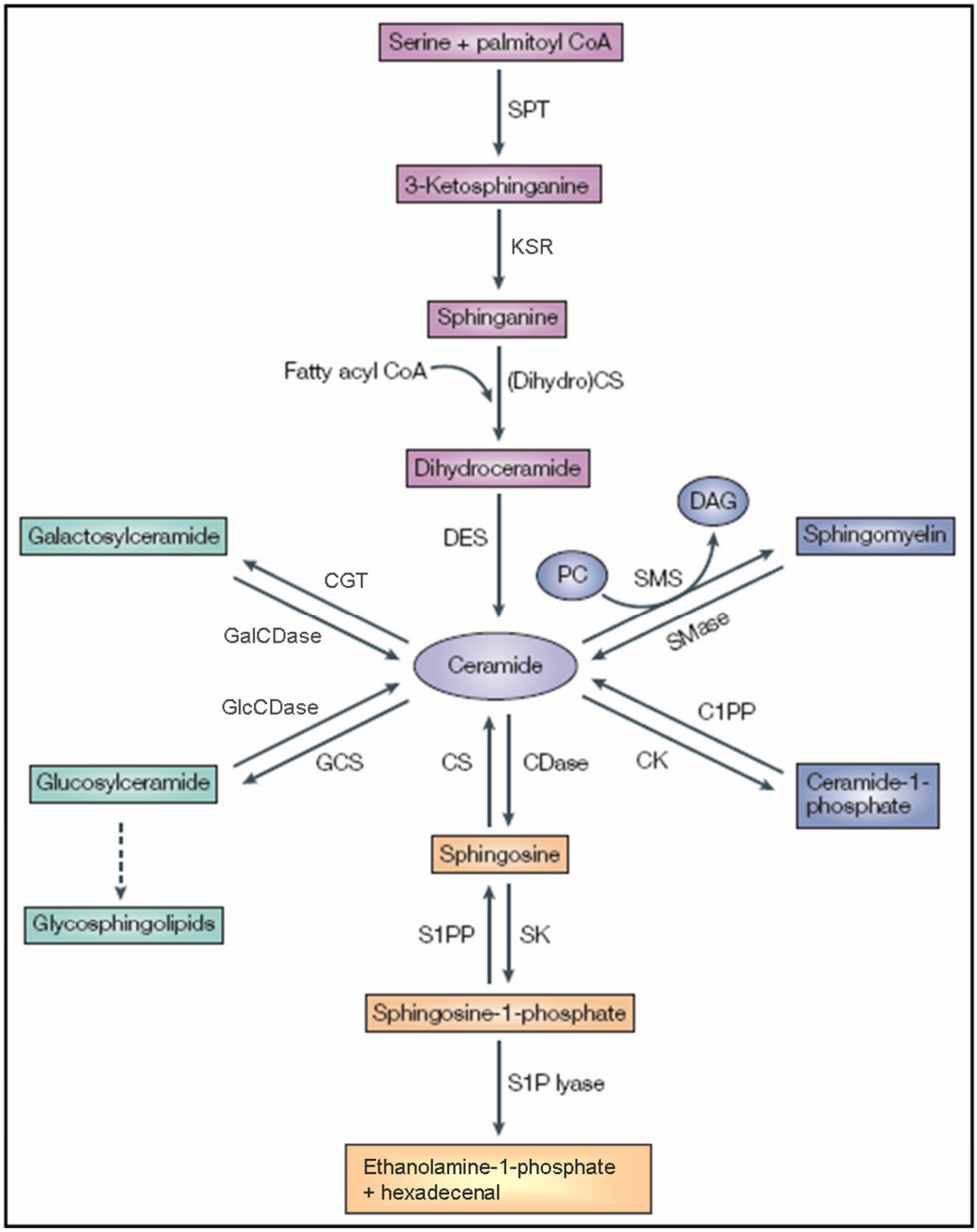

Figure 7. Schematic representation of sphingolipid metabolism (modified image from Ogretmen et al. [107]).

SPT, serine-palmitoyl transferase; KSR, ketosphinganine reductase; CS, ceramide synthase; DES, dihydroceramide desaturase; GCS, glucosylceramide synthase; GlcCDase, glucosylceramidase; CGT, ceramide galactosyl transferase; GalCDase, galactosylceramidase; C1PP, ceramide-1-phosphate phosphatase; CK, ceramide kinase; SMS, sphingomyelin synthase; PC, phosphatidylcholine; DAG, diacylglycerol; SMase, spingomyelinase; SK, sphinosine kinase; S1PP, S1P phosphatase.

2.2 Sphingolipids: signal transduction molecules

As described above, sphingolipids are ubiquitous membrane components, particularly abundant in the plasma membrane. In this context, sphingolipids physico-chemical properties enable them to fulfil and regulate a wide spectrum of relevant biological functions including molecular sorting, cell-cell interaction and intracellular transport. Indeed, membrane sphingolipids through their hydrophilic portion are able to recognize and interact with elements of the extracellular microenvironment (protein or oligosaccharide component of other cells), allowing the transfer of the information in the intracellular compartment.

Furthermore, different stimuli applied to the cells (UV radiation, chemotherapy, growth factors, etc.) generate metabolic reactions that lead to the production of different intermediate of sphingolipid metabolism (Sph, S1P, Cer, GlcCer and Cer1P), acting as bioactive molecules involved, as intra- or extra-cellular messengers, in the regulation of crucial processes as cell growth, death, migration and senescence [83].

Sphingolipid signalling is a very complex and sophisticated network of cellular pathways highly regulated and interconnected to each other. Indeed, sphingolipid metabolism can be modulated by a large number of activators, and different metabolic pathways, even activated by the same stimulus, are involved in the production of the bioactive sphingoid molecules. In addition, the sphingolipid pathways also interact with other cellular signalling systems; these interactions can result in activation, synergism or antagonism.

Enzymes involved in the sphingolipid metabolism are closely related, generating a complex interconnected network that serves not only to regulate the levels of individual bioactive lipids, but also their metabolic interconversion. The regulation of one single process can lead to variations in the levels of molecules that possess opposite biological functions. So the single bioactive molecule levels are relevant, but the balance between the levels of different molecules is more important.

This complexity is also enhanced by the different isoforms and the different subcellular localization of the enzymes involved in the sphingolipid metabolism. In addition, sphingolipids have a poor solubility in the cytosol, due to their hydrophobic nature, therefore transport mechanisms are necessary to move, transfer an finally locate the different sphingolipids in a specific subcellular site (where the enzymes of their synthesis and degradation and potential targets involved in sphingolipid-mediated signalling are located) or in the extracellular milieu. Therefore intracellular levels of bioactive sphingolipids and, consequently, the effects on biological response depend not only from the functioning and location of key enzymes of their metabolism, but also from transport mechanisms [82,83].

Sphingolipid signalling involves different types of sphingoid biomediators including S1P and Cer identified as important regulators of numerous cellular processes.

Cer and S1P emerged as critical modulators of cell survival and death. It has been shown that several growth factors stimulate the production of S1P which functions as a mitogenic molecule, favouring proliferation and cell survival [108]. On the other hand, it has been demonstrated that many stress agents can increase endogenous Cer, but also Sph levels, which in turn mediate anti-proliferative responses inducing cell cycle arrest and apoptosis [109,110].

The evidence that these molecules are interconvertible with each other and exert opposing effects on cell survival gave birth to the concept of the sphingolipid rheostat, which postulated that the dynamic ratio between ceramide toward S1P determines cell fate (Fig. 8) [111].

Given the role of the sphingolipid rheostat in regulating growth and death, it is not surprising that sphingolipid metabolism is often found to be deregulated in cancer, a disease characterized by enhanced cell growth and reduced cell death. A growing body of evidence has implicated Cer, S1P and the genes involved in their synthesis, catabolism and signalling in various aspects of oncogenesis, cancer progression and drug- and radiation resistance. In relation to their influence over cell fate, Cer and S1P are considered as tumour-suppressor and a tumour-promoting lipid respectively [107,112].

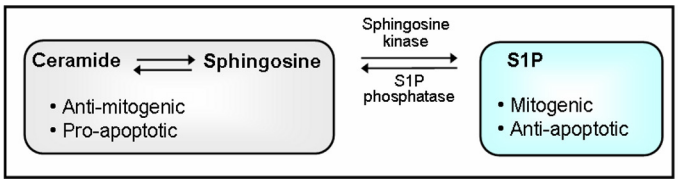

Figure 8. The sphingolipid rehostat

2.3 Cancer-suppressing role of Ceramide

Cer is known to play an important role in the evolution of neoplasia, being intimately involved in the regulation of mechanisms that control growth arrest, differentiation, senescence and cell death [109,113].

Many anticancer drugs, such as etoposide, cannabinoids and daunorubicin [114-116], and other stress-causing agonists, such as inducers of apoptosis, like FAS ligands [117], cause an increase of the endogenous Cer levels through the *de novo* synthesis. Furthermore the tumour necrosis factor α (TNF-α) in breast cancer cells [118] and ethanol in hepatoma cells [119], cause an increase in the endogenous Cer levels through the induction of the SM hydrolysis by SMases.

Of interest, the accumulation of reactive oxygen species (ROS) induces activation of acid SMase, linking oxidative stress to Cer formation [120]. In this context, Santana and co-workers demonstrated that mice deficient in acid SMase lose the ability to accumulate Cer and acquire resistance to radiation-induced apoptosis [121].

On the other hand, decreased levels of endogenous Cer caused by an increased expression of GCS, which reduces Cer levels by incorporating it into GlcCer, results in the development of a multidrug resistance phenotype in many cancer cells [122].

Cer act as an intracellular messenger regulating many intracellular effectors that mediate the activation of the apoptotic process. In particular, Cer is able to activate the serine/threonine protein phosphatases PP1 and PP2A. These phosphatases act on several substrates such as retinoblastoma protein (pRB), Bcl-2, c-JUN, PKCα, SR proteins and Akt which are all implicated in cell pathways regulating proliferation and apoptosis. Cer-mediated activation of PP1 seems to be involved in the cell cycle arrest in the G1 phase, due to the dephosphorylation of pRB [107,123]. Furthermore PP1 induces the dephosphorylation of SR proteins, a family of serine/arginine-domain proteins and known modulators of mRNA splicing, thus inducing the alternative splicing of the genes encoding Bcl-X and caspase-9, generating pro-apoptotic splice variants [117,124]. Mitochondrial membrane potential can also be altered by Cer, probably through the PP2A-mediated dephosphorylation of Bcl-2, causing its inactivation and thus favouring the apoptotic process [125]. Cer also activates Cathepsin D protease which in turn recruits and activates, via proteolytic breakdown, the pro-apoptotic protein BID resulting in the induction of apoptosis [107].

Another important Cer target is PKC-ζ. Increased levels of Cer induce the activation of this protein, by phosphorylation. The activated PKC-ζ, in turn phosphorylates the hnRNP A1 factor, involved in the mechanisms of alternative splicing. This factor might be involved in the alternative splicing of genes involved in apoptosis [110].

Cer can also exert an antiproliferative role through the inhibition of MAPK pathway, promoting the dephosphorylation of ERK1/2 (extracellular signal regulated protein kinase 1 and 2), serine/threonine kinases belonging to the family of MAP kinase (MAPK) [126]. These proteins, if dephosphorylated, are inactive and thus not able to migrate into the nucleus to promote the expression of genes involved in cell proliferation [127,128]. ERK1/2 inhibition induced by Cer can be associated with the ability of this mediator to activate, in systems "cell free", the serine/threonine protein phosphatase PP2A [129-131].

Cer can self-associate in the plane of the membrane bilayer and then fuse with GSLs- and cholesterol-containing rafts resulting in the formation of signalling platforms [132]. Many stimuli activating acid SMase at the plasma membrane induce the formation of Cer-enriched domain that trap and cluster signalling proteins [132]. In these domains the initial signal can be amplified via concentration and oligomerization of proteins that transmit signals across the plasma membrane. Such clustering has been shown for proteins associated with apoptotic signalling. Fas receptors, Fas-associated death domain-containing protein (FADD) and caspase-8 have been shown to cluster within Cer-enriched domain [133]. Furthermore, Cer can induce apoptosis by forming membrane channels in mitocondria which are large enough to cytochrome c release [134].

2.4 Ceramide in glioblastomas

Cer is known to play an important role in the evolution of neoplastic tumours, being involved as a mediator in the signal transduction mechanisms that control growth arrest, differentiation, senescence and cell death.

In gliomas the involvement of an altered regulation of the Cer-mediated signalling pathways is strongly supported by the evidence of an inverse association between Cer intracellular levels and the progression of the tumour malignancy: a lower content of Cer corresponds to a higher grade of malignancy. It was also demonstrated that Cer levels are directly related to patient survival: a low intracellular Cer content is a negative prognostic factor [135].

The metabolic pathways involved in the control of Cer levels in glial cells include enzymes of its metabolism (SMS, SPT, SMases) and its transport from the ER to the Golgi apparatus for the biosynthesis of complex sphingolipids.

It has been demonstrated that, in glioma cells, the administration of Δ9-tetrahydrocannabinol, the main active ingredient in marijuana, induces an accumulation of Cer in the ER through the stimulation of the "de novo" synthesis, resulting in apoptotic cell death [136]. Furthermore, the anti-proliferative effect of nitric oxide in

glioma cells is associated to the inhibition of Cer vesicular transport from the ER to the Golgi apparatus [137]. Of interest, in glioma cells the PI3K/Akt signalling is able to regulate the sphingolipid metabolism promoting Cer vesicular transport, resulting in a reduction of the intracellular levels of this molecule and an increased synthesis of complex sphingolipids [138].

Moreover, Cer-mediated caspase activation has been implicated in the γ-radiation-induced apoptosis in U87MG glioma cells lacking functional p53 [139]. In the same cell line, TNF-α activates both neutral and acid SMases in a p53-mediated ROS-dependent or -independent pathway that results in Cer production, which, in turn, induces apoptotic cell death [140].

Interestingly, Cer induces not only apoptosis but also caspase-independent and/or non-apoptotic cell death in glioma cells. It has been demonstrated that administration of C2-Cer results in non-apoptotic cell death induction that can be inhibited by the constitutive active form of Akt [141]. It has been shown that C2-Cer induced cell death via autophagic mechanisms [142] and natural Cer has been recently implicated in cannabinoid-induced and in TMZ-induced autophagic death in U87MG and T98G glioblastoma cells, respectively [143].

All this evidence suggests that the modulation of Cer intracellular levels can be crucial in determining the survival or death of the glioma cells. Many anticancer drugs cause an increase of the intracellular Cer levels. Consequently, tumour cells that possess an aberrant Cer metabolism develop resistance to these cytotoxic treatments, thereby determining their clinical failure.

3. SPHINGOSINE-1-PHOSPHATE

In just over one decade the sphingoid molecule S1P has emerged as a key regulator of numerous physiological functions [144]. S1P is an intermediate of sphingolipid metabolism and its levels inside cells are finely regulated through the modulation of different enzymes responsible for its synthesis and degradation [145]. S1P is formed from sphingosine (Sph) and ATP in a reaction catalyzed by two isoenzymes, sphingosine kinase 1 (SK1) and sphingosine kinase 2 (SK2) [146]. As described above, S1P can be metabolized through two different pathways: i) the dephosphorylation back to Sph, catalyzed by either lipid phosphate phosphatases or S1P specific phosphatases [103,104]; ii) the irreversible cleavage by S1P lyase, to hexadecenal and phosphoethanolamine [102].

Although a simple molecule in structure (Fig. 9), S1P functions are complex. In particular, different studies, performed in both cell cultures and animals, demonstrate that it can regulate different physiological and pathological processes strictly related to cancer and inflammation, favouring cell growth, survival, migration, and angiogenesis [82,144]. S1P can exerts its bioactive properties intracellularly, acting as a second messenger, and, mainly in the extracellular milieu, where, after secretion from cells, it acts as a ligand for specific cell surface receptors [147].

Relatively high concentrations of S1P are constitutively present in body fluids, while lower levels are detectable in tissues [148]. In particular, S1P is abundant in serum (300-500 nM) where it is mainly associated with serum proteins, such as lipoproteins (high density lipoprotein) and albumin [149].

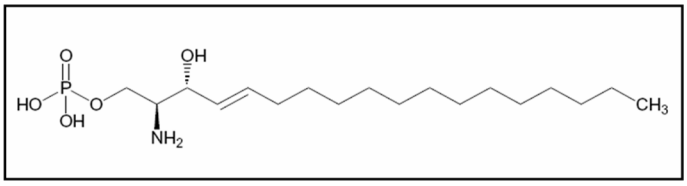

Figure 9. S1P chemical structure [145].

3.1 Sphingosine kinases

S1P is formed from sphingosine and ATP in a reaction catayzed by the enzyme sphingosine kinase (SK). So far two isoenzymes, known as SK1 and SK2, have been identified in mammals.

Both SKs are members of the diacylglycerol kinase family, containing five conserved domains responsible for substrate binding and catalytic activity. This structural homology is accompanied by SK1 and SK2 ability to catalyze the synthesis of the same product, S1P. Despite this evidence, SK1 and SK2 display different subcellular localization, tissue distribution and temporal expression pattern during development [146].

In humans, the gene coding for SK1 is located on the chromosome 17 (17q25.2), while the gene for SK2 is located on chromosome 19 (19q13.2) [151]. SK1 and SK2 are highly homologous (Fig. 10) and both contain five conserved domains (C1-C5), with the catalytic site located within the domains C1-C3 and the ATP binding site in the C2 domain. Although SK1 and SK2 display an 80% amino acid sequence similarity, they differ in their central region and N-terminus. SK1 lacks transmembrane domains or identifiable signal sequences and is mainly cytosolic. SK2 is about 240 amino acids longer than SK1 at its N-terminus and contains several transmembrane domains. In addition, SK2 possesses a nuclear localization signal in the N-terminal region, which if mutated, prevents it from entering the nucleus and inhibiting DNA synthesis [151,152]. Furthermore, both SKs have a broad and different tissue distribution, with SK1 mainly expressed in lungs, brain and spleen and SK2 predominating in the heart, brain and liver [146].

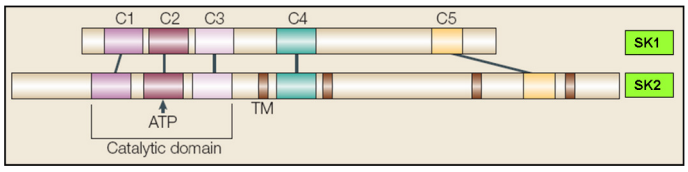

Figure 10. SK1 and SK2 structure [150].

In addition, analysis carried out by northern blot showed a different SK1 and SK2 expression during the development process, in particular SK1 transcript is the first to be encoded, while only thereafter SK2 transcript is observed [151].

It has been demonstrated that both SKs have alternative spliced variants differing in their N-terminus. In particular SK1 has three alternative isoforms (SK1a,b,c), all with similar enzymatic properties and consisting

of 384, 398 and 470 amino acids, respectively. Also SK2 has two spliced variants, the N-terminally extended (SK2-L) and the smaller (SK2-S), composed of 618 aa and 654 amino acids, respectively [153].

Finally, SK1 and SK2 differ also in the substrate affinity. Indeed SK1 has a higher affinity to D-erythro-Sph (deriving from the "recicling" sphingolipid biosynthesis); on the contrary, SK2 has a higher affinity to D-erythro-dhSph (deriving from the "de novo" sphingolipid biosynthesis). Furthermore, SK2 has a broader substrate specificity as it can also phosphorylate: phytosphingosine, DL-treo-dhSph (a SK1 inhibitor) as well as the immunomodulatory Sph analogue FTY720 [153,154].

The K_m of the human SphK-1 and SphK-2 for D-erythro-Sph are comparable (15.6 mM and 13.8 mM respectively), and their pH optima are in the neutral range [149,153].

3.2 Regulation and functional roles of SKs

Several extracellular stimuli are able to stimulate SK1 activation. In particular growth factors such as PDGF, EGF, VEGF, bFGF, IGF -1, nerve growth factor (NGF), TGF-β, cytokines such as TNF-α, interleukins and hormones play a key role in this process [151].

These molecules activate SK1 by inducing its phosphorylation on Ser225 residue in a PKC- and ERK-dependent manner. This leads to to SK1 translocation from the cytosolic compartment to the plasma membrane. In particular, the Ser225 phosphorylation causes a SK1 conformation change with the consequent exposure of the amino acids Asn84 and Thr54 present in the membrane binding domain, allowing SK1 binding to phosphatidic acid (PA) and phosphatidylserine (PS) located in the the cytosolic side of the plasma membrane [82,155-157]. Furthermore it has been demonstrated that SK1 conformational change allows its interaction with cytoskeletal proteins, such as Filamin A, which facilitates the process of re-localization of the enzyme [158]. Moreover, it has been recently shown that, once phosphorylated, SK1 relocalizes in particular portions of the plasma membrane called "lipid rafts" [158]. The localization in the plasma membrane allows SK1 interaction with its substrate, Sph, which is localized mainly at the level of the inner sheet of the plasma membrane [159]. Thus SK1 translocation to the plasma membrane is essential for Sph phosphorylation and consequent generation of S1P.

Much less is known about SK2. Its activation mechanisms are not fully understood. It has been reported that in mast cells SK2 can be activated upon crosslinking with the high affinity IgE receptor (FcεRI), or with the Src protein tyrosine kinase Fyn [158,160,161]. Of note, also EGF is able to activate SK2 and induces phosphorylation on Thr578 and Ser351 amino acid residues, both dependent on ERK 1 [158,162].

SK2 localizes primarily in the nucleus, where it is involved in cell cycle arrest. Of interest, SK2 overexpression suppresses growth and enhances apoptosis, preceded by cytochrome c release and activation of caspase-3 [163]. Indeed, SK2 contains a BH3 domain that sequesters Bcl-X_L, and abrogates its anti-apoptotic function. Therefore, SK2 can be pro-apoptotic [164].

However, the role of SK2 remains controversial, as it has been reported that SK2 protects MCF-7 breast cancer cells and HCT116 colon cancer cells from doxorubicin-induced apoptosis by a pathway mediated by p53-independent up-regulation of p21 [165]. SK2 plays a crucial role also in the regulation of the immune system response. The immunosuppressive Sph analogue FTY720 is phosphorylated in vivo primarily by SK2 with the generation of FTY720-P. This compound inhibits the outcome of T lymphocytes from the secondary lymphoid organs, desensitizing S1P receptor $S1P_1$ [159,166].

An increasing number of evidence demonstrates that SKs enzymes can be released from cells in the extracellular microenvironment; in this way it is possible to observe S1P production outside the cell.

It has been reported that SK1 release occurs both in normal and in neoplastic cells. An active form of SK1 is constitutively released by vascular endothelial cells, contributing to the vascular S1P endothelial gradient. The mechanism of SK1 release seems to occur via non classical pathway independent of the ER/Golgi system, but one that requires functional actin dynamics [167,168]. Rigogliuso and colleagues demonstrated that SK1 can be released also by cells derived from human hepatocarcinoma and human breast carcinoma as component of membrane vescicles [169]. However, the precise mechanism by which SK1 is released in the extracellular milieu remains controversial and is still under investigation.

SK2 release has been observed during apoptosis [170]. In particular, during human T cells, monocytes and human embryonal kidney (HEK293) cell death, an enzymatically active SK2 is released upon N-terminal truncation, a process demanding caspase-1 activation. Since S1P is vital for lymphocyte recruitment, Weigert an colleagues propose apoptotic cells as a local source for S1P, fundamental lipid mediator that affects immune cell attraction and activation.

Once in the extracellular environment, both SK enzymes appear to use as substrates the Sph resulting from the degradation of SM present in the extracellular leaflet of the plasma membrane, and the ATP released by cells [171].

3.3 Intracellular S1P

Despite the S1P role as an extracellular mediator is well defined, little is known about the S1P role in the intracellular compartment. S1P can exert its bioactive properties also intracellularly, acting as a second messenger. However, intracellular S1P targets are still unidentified. It has been demonstrated an involvement of S1P in the induction of cell proliferation and suppression of the cell death process independently of S1P receptors. Interestingly, microinjection of S1P into fibroblasts increases calcium mobilization from internal stores, DNA synthesis, enhancing proliferation and survival [172]. S1P role as an intracellular messenger has been also suggested by some indirect evidences, such as the PDGF-induced translocation of SK to the nuclear membrane with the concomitant increase in the nucleus-associated SK activity [147]. This implies that S1P may have a role in the nucleus, and it was suggested that it may be involved in cell cycle progression, although no direct evidence for this has yet appeared. S1P has also been shown to activate ERK and inhibit c-Jun N-terminal kinase (JNK) activation, which is significant since the balance of ERK and JNK activation has been implicated in the control of apoptosis [147].

Overall, and in contrast to the well-established actions of S1P through its receptors, the intracellular roles of S1P are poorly documented, and the identification of its direct intracellular targets remains elusive.

3.4 Extracellular S1P: release and receptors

S1P exerts its properties of bioactive molecule predominantly in the extracellular milieu. Of interest, it has been reported that S1P can be released especially from blood cells, such as platelets, red blood cells, neutrophils, mast cells and vascular endothelial cells [173]. Intriguingly, evidence on the constitutive export of S1P by cerebellar granule cells and astrocytes [174,175] supports the view that also nervous system cells can be an origin of S1P in the extracellular milieu.

Intracellularly synthesized S1P is not able to permeate the plasma membrane due to its polar nature. This evidence suggests the need for active transport mechanisms. Although the mechanism of S1P release from cells is not completely understood, recent studies have demonstrated that S1P can be released by the ATP-binding cassette (ABC) family transporters [86,173]. This family of membrane proteins is characterized by the presence of two transmembrane domains, each consisting of 6-11 membrane spanning α-helix, and two nucleotide binding domains (NBD), exposed to the cytoplasm. The two transmembrane domains define a channel through the plasma membrane. After its binding to the NBD, ATP provides the energy required to actively transport substrates across the membrane.

In the human genome 49 genes coding ABC transporters have been identified. On the basis of their sequence homology and NBD organization, human ABC transporters have been classified into seven subfamilies, designated A-G. ABC proteins have been generally recognized as drug efflux pumps that protect the body from various toxic substances, thus being implicated in the onset of drug resistance in different types of cancer. However they exhibit scarce selectivity and are able to move various molecules against their concentration gradient [176]. Increasing evidence supports that different subfamilies of ABC transporters are involved in sphingolipids passage across the plasma membrane.

In particular ABCG2, ABCA1 and ABCC1 have been implicated in the S1P transport to the extracellular milieu in breast cancer cells, astrocytes, platelets, mast cells and vascular endothelial cells [173,177-180]. Despite this evidence the precise mechanism underlying S1P secretion is not yet well defined and is still under investigation.

Once released, S1P can interact, in an autocrine and/or paracrine manner, with five specific transmembrane receptors ($S1P_{1-5}$) coupled to different G-proteins and displaying tissue-specific expression patterns [181]. Through this interaction S1P can activate several signal transduction pathways, and thus elicit a variety of cell-specific responses controlling cell behaviour [166].

The transcript for $S1P_1$ receptor has been cloned as an immediate-early gene induced during differentiation of human endothelial cells into capillary-like tubules, an *in vitro* model of angiogenesis. Thus, S1P receptors have been initially named EGD (Endothelial Differentiation Gene) receptors [182]. All the five receptors bind S1P (and dhS1P) with high affinity. Unphosphorylated Sph, and its derivates (Sph, DHSph or Cer) are unable to compete with the binding of S1P [183].

The S1P receptors are ubiquitously, but differently expressed in all cells, and are coupled to a variety of heterotrimeric G protein. The cell type specific expression of S1P receptors, as well as their differential coupling to different G proteins, contribute to the S1P ability of regulating different cellular processes in a highly specific manner.

The $S1P_1$ and $S1P_4$ couple mainly to G_i; both $S1P_2$ and $S1P_3$ activate Gi, Gq and $G_{12/13}$; $S1P_5$ is linked to G_i and $G_{12/13}$ [150]. The activation of these G proteins results in the modulation of different signalling pathway such as ERK, c-Jun N-terminal kinase (JNK), the small GTPases of the Rho family (Rho and Rac), phospholipase C (PLC), adenylate cyclase-cAMP, and PI3K/Akt signalling [149,150,166] (Fig. 11). Through its receptors, S1P is able to regulate different cellular processes, such as cell proliferation, motility, invasion, cytoskeletal rearrangement, angiogenesis, vascular maturation and lymphocyte trafficking and actions [149,150].

$S1P_1$ is ubiquitously expressed, with high levels in brain, lungs, spleen, heart and kidneys. It plays a key role in angiogenesis and vascular maturation, regulation of immune cell trafficking, endothelial barrier function, and vascular tone. It has been demonstrated that, upon binding with S1P, $S1P_1$ is able to trans-activate growth factor receptor tyrosine kinase (RTKs), such as VEGF, PDGF, and EGF receptors. This can occur through three distinct mechanisms: i) intracellular cross-talk and direct phosphorylation of RTK by protein tyrosine kinases; ii) induced production and/or secretion of growth factors; iii) participation of $S1P_1$ and RTK in a signal complex, either by direct interactions or by binding of both receptors to scaffold proteins [149,150].

$S1P_2$ is widely expressed in a variety of different cell types. It plays fundamental roles in the cardiac development, and in the development and maintenance of neuronal excitability [149]. Furthermore, this receptor is required for proper development of the auditory and vestibular systems, and for proper degranulation of mast cells. $S1P_2$ can exert a negative role in the regulation of cell migration, and overexpression of $S1P_2$ is able to inhibit S1P-induced cell migration [184]. For this reason it is considered to be a repellent S1P receptor, and appears to work in opposition to $S1P_1$ and $S1P_3$, both enhancing cell migration [185]. Finally, $S1P_2$ activation leads to an increased vascular permeability through ROCK/Rho activation [181].

$S1P_3$ is abundantly expressed in the cardiovascular system, lungs, spleen, kidneys, intestines and cartilage. This receptor is an important regulator of vascular permeability through the downstream effectors ROCK and Rho proteins. A clear role for $S1P_3$ has also been demonstrated in the regulation of the heart rate, as its expression is localized in myocytes and perivascular smooth muscle cells, and its activation results in bradycardia and hypertension. Furthermore, S1P binding to this receptor has been shown to enhance cell survival by suppression of Bax expression and activation of endothelial NOS, PI3K and Akt [147,181].

$S1P_4$ is primarily expressed in lymphoid tissues, including thymus, spleen, bone marrow and peripheral leukocytes. $S1P_4$ stimulation activates ERK1/2, phospholipase C and modulates the opening of intracellular calcium stores. This receptor seems to be implicated in the regulation of T-cell migration and cytokine production [149,186].

$S1P_5$ is highly expressed in the brain, in particular in oligodendrocytes. The binding to S1P induces a phosphatase dependent inhibition of ERK 1/2, resulting in an antiproliferative phenotype. Recently it has been demonstrated that this receptor is present in Natural Killer cells (NK) and is involved in the homing and mobilization of these cells to inflamed organs [147,186].

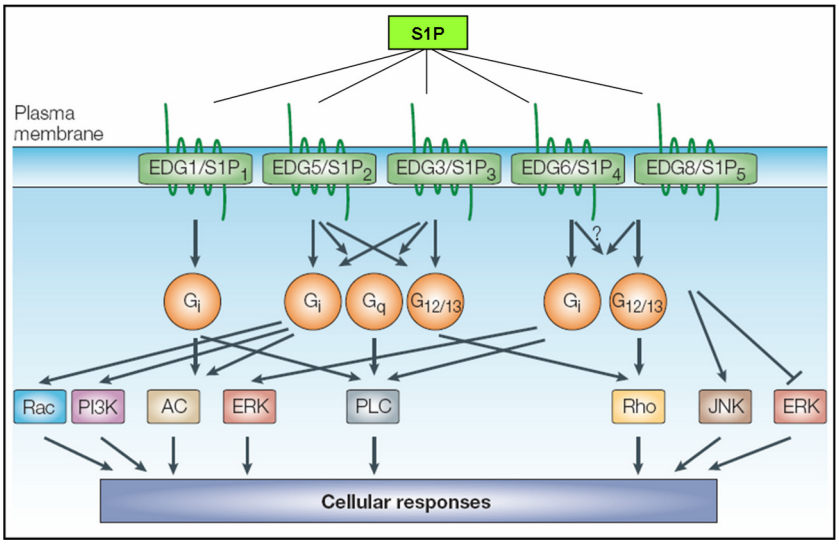

Figure 11. Signal transduction pathways activated by S1P receptors (modified image from Spiegel et al. [150])

AC, adenylate cyclase; JNK, c-Jun N-terminal kinase; PLC, phospholipase C.

3.5 Cancer-promoting roles of S1P

Cancer is a disease characterized by a total alteration of normal cellular processes that govern cell proliferation and normal cellular homeostasis. These alterations allow tumour cells to acquire a malignant phenotype and survival advantages.

A growing body of evidence has implicated S1P and SK1 in various aspects of oncogenesis, both favouring cell survival, migration, angiogenesis and drug resistance, thus identifying a fundamental role of these two molecules in the onset of cancer (Fig. 12) [107,187].

Cell survival

SK1 has emerged as an oncogene, found overexpressed in multiple types of cancer including brain, colon, lung and kidney tumours, compared with their healthy counterparts and associated with tumour grading as well as reduced patient survival [188,189]. In agreement, the down-regulation of SK1 expression in cancer cells reduces growth and increases apoptosis [190]. Xia and colleagues [191,192] demonstrated that SK1 overexpression in murine 3T3 fibroblasts causes cell transformation in culture and tumour onset in SCID mice. Moreover, SK1 overexpression in MCF-7 breast cancer cells increases cell proliferation and DNA synthesis, accelerates G1/S transition of the cell cycle, and enhances estrogen-dependent tumorigenesis [193]. In addition, SK1 overexpression in non-tumorigenic pro-erythroblasts increased their clonogenicity as

well as resistance to apoptosis, suggesting that high expression of SK1 may be an oncogenic event required for progression of erythroleukemia [194].

S1P has emerged as an onco-promoter molecule. Interestingly, the administration of S1P to different cell types results in the inhibition of different forms of cellular apoptosis, including the Cer-mediated one [111], through the induction of pro-survival proteins expression (Bcl-2, MCL1), stimulation of the DNA replication, and inhibition of pro-apoptotic proteins expression (Bad, Bax). Exogenous S1P also blocks the translocation of Bax to the mitochondria, which is required for the release of mitochondrial cytochrome c and the consequent caspase activation [187].

S1P was also shown to promote estrogen-dependent tumorigenesis of MCF-7 human breast cancer cells [193]. Johnson and colleagues used siRNAs to show that inhibition of S1P phosphatase 1 (S1PP1), which converts S1P to sphingosine, results in increased intracellular and extracellular levels of S1P and endows MCF-7 cells with resistance to the cytotoxic actions of TNFα and daunorubicin [195].

Cell motility

S1P stimulates the motility of cancer cells through $S1P_1$ or $S1P_3$. By contrast, S1P can inhibit cancer cell motility through $S1P_2$-dependent regulation of Rho. The specific effect of S1P is partly determined by the predominance of the receptor subtypes expressed. For example, S1P stimulates the migration of gastric tumour cells that exclusively expressing $S1P_3$ and inhibits the motility of others that predominantly express $S1PR_2$ [196].

Similarly, the inhibition of melanoma cell migration is through $S1P_2$ and involves the inhibition of Rac, activation of Rho, and the subsequent tyrosine phosphorylation of focal adhesion kinase and paxillin as well as the increment of stress fibre formation [197,198].

On the other hand, S1P stimulates the migration of fibrosarcoma cells through a $S1P_1$–Rac1–CDC42-dependent pathway involving the tyrosine phosphorylation of membrane type matrix metalloproteinase 1 (MT1-MMP), a zinc-dependent proteolytic enzyme involved in degradation of the extracellular matrix, facilitating cell migration [199,200]. Furthermore, ovarian cancer cell invasion induced by S1P involves $S1PR_1$ or $S1PR_3$ and calcium mobilization [201].

Of interest, the pharmacological inhibition of both SKs suppresses chemotaxis following growth factor stimulation in diverse cell types [188]. In particular, in breast cancer cells the down-regulation of both SK1 and SK2, suppresses EGF-induced migration, while overexpression of either SK1 or SK2 enhances migration towards EGF [202]. A link between S1P signalling, cell migration and tumour metastasis was

uncovered when it was observed that the overexpression of the KAI1 gene down-regulates SK1. The KAI1 gene was originally isolated as a prostate-specific tumour metastasis suppressor gene that inhibited metastases without affecting primary tumour formation. The KAI1 overexpression in pancreatic carcinoma cells significantly reduced both SK1 activity and cell migration [203].

Tumour angiogenesis

Angiogenesis plays a key role in providing oxygen and nutrients to the tumour mass, as well as a direct access to the bloodstream, thus facilitating the generation of metastasis. An increasing number of evidence demonstrated that S1P acts as a potent pro-angiogenic factor, inducing the migration of endothelial cells and promoting the formation of new blood vessels.

The mechanisms of S1P-mediated neovascularization involve the migration of endothelial cells through the activation of S1P receptors, and downstream regulation of the RHO family of small GTPases, which in turn regulate cell motility and remodelling of the cytoskeleton [204]. The main S1P receptor involved in the process of vascular maturation is $S1P_1$, which is required for the stabilization of nascent blood vessels during embryonic development, indeed its knockout in mouse resulted in a lethal vascular defect [205].

$S1P_1$ expression is enhanced in lung carcinoma vasculature during angiogenesis, and its knockdown inhibits endothelial cell migration *in vitro* and the growth of neovessels into subcutaneous implants of Matrigel *in vivo,* resulting in the dramatic suppression of tumour growth [206]. These evidences strongly suggest that $S1P_1$ is a crucial receptor in mediating angiogenesis and metastasis in tumours.

There is also evidence that SK1 is involved in angiogenesis. Indeed, SK1 overexpression in endothelial cells enhances survival after serum deprivation and detachment from the extracellular matrix, suggesting that it may play an important role in vascular phenomena that occur under stress conditions [207].

It has been demonstrated that there is a crosstalk between SK1 and the VEGF signalling, which is crucial for angiogenesis. In particular, VEGF was shown to stimulate SK1 activity in bladder tumour cells and, in turn, SK1-mediated VEGF-induced activation of Ras and MAPKs. Indeed, SK1 inhibition reduces VEGF-stimulated MAPK activation in in these cancer cells [208]. As S1P is also secreted extracellularly, modulation of SK and S1P in tumour cells provides a potential mechanism for recruiting endothelial cells and promoting blood-vessel formation/angiogenesis [107]. Indeed, combining S1P with other pro-angiogenic factors, such as basic fibroblast growth factor or VEGF, produced synergistic enhancement of neovascularization in tissue samples of mouse aortic rings, an *ex vivo* model of angiogenesis [209].

Drug resistance

One of the main obstacles involved in cancer therapy is the development of drug resistance. A significant body of literature now implicates SK1/S1P signalling in this process, since it protects cancer cells from chemotherapy-induced apoptosis. For example, in prostate adenocarcinoma SK1 regulates drug-induced apoptosis and serves as a chemotherapy sensor both in culture and in animal models. In particular, SK1 overexpression impaired the efficacy of chemotherapy (Docetaxel and Camptothecin). Alternatively, silencing SK1 by RNA interference or pharmacologic inhibition induced apoptosis both *in vitro* and *in vivo* [210].

In parallel with these data, increasing the expression of SK1, reduced the sensitivity of melanoma cells to Fas- and Cer-mediated apoptosis that could be reversed by the inhibition of SK1 expression [211].

Furthermore, high expression levels of SK1 and S1PRs were detected in Camptothecin- (CPT) resistant prostate cancer cells. Specifically, inhibition of SK1 expression or S1PR signalling significantly inhibited cell growth, and treatment of these cells with CPT induced upregulation of SK1/S1PR signalling [212]. Moreover, it has been demonstrated that SK1 overexpresion increases proliferation and resistance to Tamoxifen of breast cancer cells, whereas siRNA knock down of SK1 restores Tamoxifen responsiveness [213]. Of interest, S1P-lyase, which irreversibly degrades S1P, was found to play a crucial role in the increased sensitivity of human colon cancer cells to apoptotic stimuli, in a p38- and p53- dependent manner [214].

Taken together, this evidence demonstrates a relationship between the changes in the S1P metabolism and development of drug resistance in human cancer cells. Therefore, one possible approach to overcome this resistance could be through modulation of the sphingolipid pathway [187]. Strategies that could be adopted to limit the effects of S1P signalling in cancer include the pharmacological inhibition of SK and targeting of S1P receptors.

A number of novel inhibitors of human SK were identified. These compounds are antiproliferative toward a bladder and breast cancer cell lines, including those lines with the multidrug resistance phenotype, because of overexpression of either P-glycoprotein or multidrug resistance phenotype 1. Furthermore, each inhibitor induced apoptosis concomitant with tumour cell cytotoxicity [189]. The dimethylSph (DMS), a sphingosine analogue widely used as a pharmacological inhibitor for SK, inhibits leukaemia, colon, epidermoid and lung tumour cell growth and reduces metastasis *in vivo* [215,216]. DMS also increases the sensitivity of human leukaemia cells to apoptosis in response to radiation, TNF-α and FAS ligand [217]. Another Sph isomer, Safingol, induced apoptosis and increased the growth-inhibitory actions of Doxorubicin even in multidrug-

resistant cancer cells. Safingol is under evaluation in Phase I trials for the treatment of various human cancers in combination with other chemotherapeutic agents such and Cisplatin [218].

However, these compounds, without SK isoform specificity, also inhibit PKC and Cer kinase, as well cause hemolysis and hepatotoxicity [219]. Since SK1 and SK2 may have different functions in cancer progression, the specific targets for these isoforms are needed. SK1-I (BML-258), a specific SK1 inhibitor, prevents tumour growth and vascularisation, and induces apoptosis in glioblastoma xenografts. In addition, SK1-I enhances survival in orthotopic glioblastoma [220]. Recently, a SK2-selective inhibitor (ABC294640) has been identified. It inhibits tumour growth, induces apoptosis and autophagic cell death in kidney tumour xenografts [221].

It has been demonstrated that the immunosuppressant Fingolimod (FTY720), an analogue of sphingosine currently used in multiple sclerosis treatment, has also anticancer effects. In particular, it is taken up by cells, phosphorylated to FTY720-P by SK2 [154,222], and then released. Extracellular FTY720-P can bind to S1P receptors (except S1P2), causing their rapid poly-ubiquitinylation, endocytosis and proteasomal degradation, thus inducing sequestration of T lymphocytes in lymphoid tissues [223,224]. In addition to its immunosuppressive function, FTY720 was shown to induce growth arrest and apoptosis in leukemia, bladder, prostate, breast cancer, and also glioma cells [225-228]. Furthermore, FTY720 is able to prevent tumour growth and metastasis in mouse breast cancer cells, both *in vitro* and *in vivo* [229]. Of interest, FTY720 inhibits tumour vascularization and angiogenesis in vivo, in both hepatocellular carcinoma and prostate cancer, through the functional antagonism of S1P1 in endothelial cells [230-232].

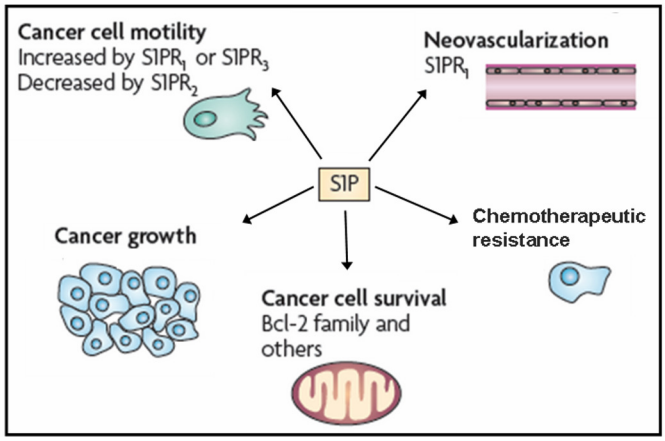

Figure 12. Summary of S1P roles in tumorigenesis (modifierd image from Pyne et al.[187]).

3.6 S1P in gliobastomas

A growing body of evidence has implicated S1P and the genes involved in its synthesis, catabolism and signalling in various aspects of oncogenesis, cancer progression and drug- and radiation resistance [107]. S1P has emerged as an onco-promoter molecule in different tumours, including GBM [173,187,233]. Notably, it has been previously documented that S1P enhances proliferation [234], motility, invasiveness and malignant behavior of GBM cells [235]. As an indication of the importance of this molecule, different studies have reported that GBM cell lines and tissue specimens show an high SK1 expression [236,237] which correlates with a worst prognosis and a poor patient survival [238] and that silencing or pharmacological inhibition of both SK1 and SK2 decreases the proliferation rate in GBM cells, preventing their entry into the cell cycle [236,238].

It has been reported that glioma cells are able not only to produce S1P, but also to release it in the extracellular milieu [237,239]. This evidence suggests that S1P may play an important role as autocrine/paracrine messenger in GBM.

Furthermore, it has been extensively demonstrated that glioblastoma cells commonly express S1P receptors, $S1P_1$, $S1P_2$, and $S1P_3$ and very low levels of $S1P_5$ [234,240,241], all contributing to GBM cell growth and invasion through distinct, but overlapping mechanisms. For example, it has been demonstrated that, through activation of $S1P_1$, S1P up-regulates the expression of urokinase plasminogen activator (uPA), a potent stimulator involved in cancer cells invasion, in human glioblastoma multiforme cells [242].

3.7 S1P in glioblastoma stem cells

Interestingly, in the last years S1P is emerging as an important mediator determining GSCs properties. Annabi and co-workers suggested for the first time that GSCs, isolated from a glioblastoma cell line, express S1P receptors (mainly $S1P_1$) and that the exogenously added S1P promotes migration and invasivity of these cells *in vitro* via membrane-type-1 matrix metallopreoteinase MT1-MMP [243]. Furthermore, Mora and colleagues demonstrated that the treatment with a SKs inhibitor causes the dissociation of neurospheres, the structures usually formed by GSCs in culture, abolishing their growth and favouring cell death [244].

Estrada-Bernal and collaborators demonstrated that GSCs expressing a constitutively active EGF receptor mutant (EGFR vIII) are characterized by high levels of SK1 activity. EGFR inhibitors modestly decrease SK1 activity and proliferation of these cells in culture. More extensive blockage of SK1 activity potently inhibits cell

proliferation and induces GSCs death. Thus SK1 activity is necessary for GSC survival, and EGFRvIII partially utilizes SK1 to further enhance cell proliferation [245].

Furthermore, a recent study demonstrated that the inhibition of S1P receptors, through the administration of FTY720, decreases GSCs viability and acts synergistically with TMZ *in vitro*. Moreover, *in vivo* FTY720 promotes survival in a rodent model of GBM and decreases GSCs invasiveness in nude mouse brains [246].

AIM OF THE WORK

GBM is the most frequent, aggressive and deadly primary central nervous system tumour in humans [1]. Despite the introduction of the alkylating agent TMZ in glioblastoma therapy has improved patient survival, drug resistance mechanisms limit its benefits and the prognosis remains unfavorable [33]. Growing evidence suggests that GSCs, a cell subpopulation within the tumour, play a crucial role in the GBM malignant behaviour, being involved in the processes of chemo-resistance, recurrence, metastasis and angiogenesis [49,50,52]. However, the precise mechanisms underlying the chemoresistance and the malignancy of GSCs are not completely understood.

A large amount of evidence demonstrates the role of S1P as an important tumour-promoting lipid, exerting its effects predominantly in the extracellular milieu after interaction with specific G protein-coupled receptors [144,173], thus favouring growth, invasion, and chemotherapy resistance of different cancer cells [247], including glioblastoma ones [235,248]. Recent pieces of evidence suggest that S1P could represent an important mediator of GSCs properties, too. Indeed GSCs do express S1P receptors and exogenously added S1P promotes migration and invasivity of these cells in vitro via MT1-MMP [243]. Furthermore, the inhibition of S1P biosynthesis results in the dissociation of neurospheres (the structures usually formed by GSCs in culture), in the arrest of proliferation, and in cell death [244,245].

Despite these promising findings, up to now the putative role of S1P as an autocrine/paracrine factor modulating GSCs survival has been poorly investigated. Notably it is still unclear if GSCs are able to produce and release their own S1P extracellularly, or if S1P secreted by other neighbour cells (such as neurons, astrocytes, endothelial cells and glioblastoma cells), is the real responsible for the S1P-mediated responses observed in GSCs. In this context, it is worth noting that neural stem cells have been shown to be unable to release S1P extracellularly, but S1P exhibits a potent chemo-attractant activity on these stem cells [249].

On these premises, the aim of this PhD project was to investigate the ability of glioblastoma cells and GSCs to produce and release S1P in the extracellular milieu. Moreover, we evaluated the possible involvement of S1P as an autocrine/paracrine factor modulating TMZ resistance, with particular attention to S1P cross-talk with ceramide, a tumour-suppressor lipid exerting opposite effects on cell survival compared to S1P [111]. The overall rationale of this research was that a better understanding of S1P role in GSC aggressive phenotype could represent a critical start point that sets the bases for the development of new compounds able to modulate S1P metabolism in order to sensitize GSCs to chemotherapeutic treatments.

MATERIALS AND METHODS

MATERIALS

All reagents were of analytical grade. DMEM, L-glutamine, penicillin, streptomycin, amphotericin B, EGF, insulin, fatty acid free bovine serum albumin (BSA-FFA), 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide (MTT), aprotinin, leupeptin, pepstatin, bestatin, Kodak Biomax film, D-erytrhro-sphingosine, C6-ceramide (N-hexanoyl-D-erythro-sphingosine), C2-ceramide (N-acetyl-D-erythro-sphingosine), bovine brain ceramide and other common chemicals were from Sigma Aldrich (St.Louis, MO, USA).

Fetal calf serum (FCS) was from EuroClone (Pero, Milan, Italy). bFGF was purchased from PeproTech (Rocky Hill, NJ, USA). B27 supplement, DMEM/F12 and Trypsin TrypLE Express were obtained from Invitrogen (Carlsbad, CA, USA). RNeasy mini kit and RNase-free DNAse I were from Qiagen (Valencia, CA, USA). iScript cDNA synthesis kit and SYBR green super mix were from Biorad Laboratories (Hercules, CA, USA). Temozolomide was from Schering-Plough (Segrate, Milan, Italy). Sphingosine-1-phosphate and primary mouse anti-ABCG2 antibody were purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Primary goat anti-MGMT antibody, primary mouse anti-ABCC1 antibody and mouse anti-goat horseradish peroxidase-linked secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary rabbit anti-SK1 and anti-SK2 antibodies were purchased from Abcam (Cambridge, UK). Primary mouse anti-ABCA1 antibody was from Chemicon-Millipore (Billerica, MA, USA). Goat anti-mouse horseradish peroxidase-linked secondary antibody was from Thermo Fisher Scientific (Waltham, MA, USA). Goat anti-rabbit horseradish peroxidase-linked secondary antibody, SuperSignal West Pico and West Femto Maximum Sensitivity Chemioluminescent Substrate were purchased from Pierce Chemical Co (Rockford, IL, USA). [^{3}H]-D-erythro-sphingosine ([^{3}H]-Sph) was from Perkin Elmer (Boston, MA, USA). [^{3}H]-dihydrosphingosine ([^{3}H]-DHSph) was from American Radiolabeled Chemicals (St Louis, MO, USA). [γ-^{32}P]ATP was from GE Healthcare (Milan, Italy). High performance thin layer chromatography (HPTLC) silica gel plates, and all solvents were purchased from Merck (Darmstadt, Germany). Sphingosine kinases inhibitor (SKI) was from Echelon Biosciences Inc. (Salt Lake City, UT, USA).

METHODS

4.1 Cell cultures

T98G glioblastoma multiforme cell line

T98G human glioblastoma multiforme cell line was cultured in a humidified atmosphere at 37° C with 5% CO_2 and expanded *in vitro* in DMEM containing: 10% (v/v) heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The culture medium was changed every 48 hours. Cells were maintained by serial passages in 75 cm^2 culture flasks. When the culture reached confluence, subculture was prepared using a 0.025% trypsin–0.01% EDTA solution. Cells were re-suspended in DMEM 10% FCS, counted by trypan blue dye exclusion test using a Neubauer emocytometer and then re-plated (13-26×10^3 cells/cm^2).

Temozolomide-resistant T98G cell line

Temozolomide-resistant (TMZ-R) cells were obtained from T98G cells by gradual and repeated treatments with increasing concentrations of TMZ between 0.1 mM and 1.5 mM for 48 hours at each passage of duplication. In particular, at each step of selection, cells were exposed to a higher drug concentration as soon as re-growth was apparent. This kind of drug administration provides a selective pressure that leads to the survival of resistant cells no longer sensitive to therapy. In these conditions, a subpopulation of cells able to grow in the presence of 1.5 mM TMZ has been selected.

Resistant cells were cultured as described for T98G cells and maintained by 24-hours exposure to 1.5 mM TMZ once every two passages of duplication.

U87-MG glioblastoma multiforme cell line

U87-MG human glioblastoma multiforme cell line was cultured in a humidified atmosphere at 37° C with 5% CO_2 and expanded *in vitro* in DMEM containing: 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The culture medium was changed every 48 hours. Cells were maintained by serial passages in 75 cm^2 culture flasks. When the culture reached confluence, subculture was prepared using a 0,025% trypsin–0.01% EDTA solution. Cells were re-suspended in DMEM 10% FCS, counted by trypan blue dye exclusion test using a Neubauer emocytometer and then re-plated (20-30×10^3 cells/cm^2).

U-SC glioblastoma stem cells

U-SC were isolated from the stable U87-MG cell line using a selective medium composed of DMEM/F12 without serum and supplemented with 10 ng/ml bFGF, 20 ng/ml EGF, 5 µg/ml insulin, B27 supplement, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. To this purpose U87-MG cells were plated in 100 mm Petri dishes at 3 x 10^3 cells/cm^2 in 5 ml of selective medium and mantained at 37°C in a humidified atmosphere of 5% CO_2 for one month.

In order to obtain the progressive enrichment and stable expansion of U-SC in culture, during the isolation process every week neurospheres (the compact cell clusters floating in suspension usually formed by GSCs in culture) were subcultured by harvesting, followed by dissociation and re-plating under the same growth conditions. To this end the culture medium containing neurospheres in suspension was centrifuged at room temperature for 10 minutes at 280 x g. Subsequently, the cellular pellet obtained was incubated with Trypsin TrypLE Express, resuspended in order to obtain the complete cell dissociation and then cells were counted by trypan blue dye exclusion test using a Neubauer emocytometer. After a centrifugation at room temperature for 10 minutes at 280 x g, cells were resuspended in selective medium and re-plated in order to keep the process of selection. The expression of brain cancer stem cell markers was then evaluated in order to test the successful enrichment in U-SC of the cell population obtained by selective isolation.

Once isolated, cells were maintained by serial passages in a humidified atmosphere at 37° C with 5% CO_2 in 75 cm^2 culture flasks, using the same culture medium used during selection. Once a week neurospheres were dissociated and counted as described above and then re-plated ($25x10^3$ cells/cm^2) to allow cell propagation.

L0627 glioblastoma stem cells

The GSC line L0627 was obtained from a post-surgery specimen of a primary human glioblastoma multiforme, as previously described [48]. L0627 were maintained at 37°C in 5% CO_2 humidified atmosphere and expanded *in vitro* in a selective medium consisting of serum-free DMEM/F12 supplemented with: 10 ng/ml bFGF, 20 ng/ml EGF, 0.6% glucose, 0.1% $NaHCO_3$, 5 mM Hepes, 2 mM L-glutamine, 0.0004% heparin, 100 units/ml penicillin, 100 µg/ml streptomycin and 10X Hormone Mix (consisting of DMEM/F12 with the addition of: 0.6% glucose, 0.1% $NaHCO_3$, 5 mM Hepes, 1 mg/ml apo-transferrin, 0.2 mg/ml insulin, 0.1 mg/ml putrescine, 0.3 µM selenium and 0.2 µM progesterone) [250].

Cells were maintained by serial passages in 75 cm^2 culture flasks and when the neurospheres reached an average diameter of 100-300 µm, subculture was prepared (every 72-96 hours). To this purpose culture

medium containing cells in suspension was centrifuged at room temperature for 5 minutes at 194 x g. Cells were then disaggregated by mechanical dissociation, counted by trypan blue dye exclusion test, resuspended in culture medium and then re-plated (20×10^3 cells/cm^2).

PT 1 and PT 2 glioblastoma stem cells

PT 1- and PT 2-GSCs were isolated at the Carlo Besta Neurological Institute, from GBM specimens obtained from two patients showing a different GBM aggressive phenotype (PT 1 and PT 2). Cells were maintained by serial passages in a humidified atmosphere at 37° C with 5% CO_2 and 93% N_2 (hypoxic conditions) in 75 cm^2 culture flasks. Cells were expanded *in vitro* in the same selective medium described for L0627 cells and when the neurospheres reached an average diameter of 100-300 μm, subculture was prepared. In particular, culture medium containing cells in suspension was centrifuged at room temperature for 10 minutes at 280 x g, cells were disaggregated by enzymatic dissociation (Trypsin TrypLE Express), counted by trypan blue dye exclusion test, resuspended in culture medium and then re-plated.

All cell models were plated for experiments at the opportune density in tissue plate dishes (3.2 cm of diameter) or in 24/96-well plates (1.5 and 0.6 cm of diameter, respectively).

4.2 RNA isolation, reverse transcription and Real-Time PCR

Total RNA was isolated from U87-MG and U-SC cells with the RNeasy mini kit and treated with the RNase-free DNAse I. One microgram of RNA was reverse transcribed using the iScript cDNA synthesis kit according to manifacturer's instructions. Real-Time PCR was performed using the iQ5 Real-Time PCR detection system (Biorad Laboratories, Hercules, CA, USA). Specific SYBR green expression assays (SYBR green super mix) for CD133, nestin and GAPDH were carried out. Simultaneous amplification of the target sequences was performed as follows: 3 minutes at 95°C, 50 cycles 95°C 10 seconds, 58°C 40 seconds, 60°C 10 seconds and 1 cycle 60°C 3 minutes. Results were analyzed using the iQ5 optical system software (Biorad Laboratories, Hercules, CA, USA). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [251]. Data were normalized to GAPDH expression (used as endogenous control) and U87-MG cells were used as calibrator.

4.3 Cell treatments

For cell treatments, stock solutions were prepared by dissolving the different molecules as follows:

- TMZ: 200 mM stock solutions were prepared in DMSO;
- C2-Cer: 25 mM stock solutions were prepared in ethanol;
- C6-Cer: 50 mM stock solutions were dissolved in ethanol;
- Bovine brain Cer: 10 mM stock solutions were prepared in Ethanol/dodecane 98:2 (v/v);
- SK inhibitor (SKI): 15 mM stock solutions were dissolved in DMSO;
- S1P: 100 μM stock solutions were prepared in fatty acid free bovine serum albumin 4mg/ml in PBS and incubated for 30' at 37°C with constant stirring before use.

Stock solutions were diluted extemporaneously in fresh medium at the desired concentrations and administered to cells for the indicated period of time. Of relevance, ethanol and DMSO final concentrations in the culture medium were always maintained below 0.5%, concentrations that do not affect cell survival. In each experiment untreated cells were incubated with vehicles at the same final concentration of treated cells as controls. At the end of the treatments cell survival was evaluated by MTT assay.

4.4 Analysis of cell viability

Cell viability was determined by MTT colorimetric assay. U87-MG and GSCs were seeded at 1 x 10^4 and 2 x 10^4 cells/cm^2 respectively in 24- or 96-well plates. The day after cells were treated with different agents for the indicated periods of time. The medium was then replaced by MTT dissolved in fresh medium (0.8 mg/ml) for 4 hours. The formazan crystals formed in viable cells were then solubilized in iso-propanol/formic acid (95:5 v/v) for 10 minutes with constant stirring to ameliorate the lysis process. The absorbance at 570 nm was measured using a microplate reader (Wallack Multilabel Counter, Perkin Elmer, Boston, MA, USA). In some cases (see text), cell viability was also determined by trypan blue exclusion.

4.5 Immunoblotting analyses

Protein expression levels were evaluated using different conditions of cell lysis, SDS-PAGE and Western blotting depending on the antigen analysed, as described below.

In all cases a small aliquot of cell lysates was analyzed for the protein content with the Comassie Blue-based assay. The remaining lysate was denatured by the addition of Sample Buffer 4X (containing 0.25 M Tris- HCl

pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.2% (w/v) bromophenol blue, 0.4 M DTT) and then proteins were resolved by SDS-PAGE and detected by Western blotting as follows.

MGMT

Cells were lysed with 20 mM tris HCl pH 8.5, 1 mM EDTA, 5% glycerol, 1 mM β-mercaptoethanol, 0.1 mM PMSF in EtOH, in presence of protease inhibitors (10 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 3 μg/ml bestatin), as recently described [252]. Cell proteins were resolved by SDS-PAGE on 12% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were then blocked in 5% milk using 0,05% PBS-Tween20, incubated for 1 hour with anti-MGMT primary antibody (1:50) and finally with a mouse anti-goat horseradish peroxidase-linked secondary antibody (1:2000). β-actin was used as loading control.

Sphingosine kinases

Cells were lysed with 20 mM HEPES pH 7.4, 50 mM NaCl,1 mM EGTA pH 8, 1% Triton X100, 5 mM β-glicerophosphate, 2 mM sodium orthovanadate, 0.1 mM sodium pyrophosphate, 1 mM EDTA, in presence of protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin). In order to evaluate SK1 expression, cell proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Moreover, to evaluate SK2 expression, cell proteins were resolved by SDS-PAGE on 12.5% polyacrylamide gels and transferred onto PVDF membranes. Membranes were then blocked in 5% milk using 0.05% TBS-Tween20, incubated for 1 hour with anti-SK1 (1:500), anti-SK2 (1:3000) primary antibodies and then with goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:500 and 1:300 respectively). GAPDH or β-actin were used as loading control.

ABCG2

Cells were lysed with 10 mM tris HCl pH 7.4, 0.25 M sucrose in presence of protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Cell proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were then blocked in 10% milk using 0.1% TBS-Tween20, incubated overnight with anti-ABCG2 primary antibody (1:1000) and finally with goat anti-mouse horseradish peroxidase-linked secondary antibody (1:2000). β-actin was used as loading control.

ABCA1

Cells were lysed with 10 mM tris HCl pH 7.4, 1 mM $MgCl_2$, 1% NP-40, 0.5% Na-deoxycholate, 5 mM EDTA in presence of protease inhibitors (1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin). Cell proteins were resolved by SDS-PAGE on 7.5% polyacrylamide gels and transferred onto PVDF membranes. Membranes were then blocked in 5% milk using 0.1% TBS-Tween20, incubated for 2 hours with anti-ABCA1 primary antibody (1:1000) and then with goat anti-mouse horseradish peroxidase-linked secondary antibody (1:1000). β-actin was used as loading control.

ABCC1

Cells were lysed with 50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 100 mM NaF, 1 mM EGTA pH 8, 10% glycerol, 1% Triton X100, 0.2 mM sodium orthovanadate, 10 mM sodium pyrophosphate in presence of protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 5 μg/ml pepstatin). Cell proteins were resolved by SDS-PAGE on 7.5% polyacrylamide gels and transferred onto PVDF membranes. Membranes were then blocked in 5% milk using 0.2% TBS-Tween20, incubated for 1 hour with anti-ABCC1 primary antibody (1:250) and finally with goat anti-mouse horseradish peroxidase-linked secondary antibody (1:1000). β-actin was used as loading control.

In all cases bound antibodies were visualized by ECL (SuperSignal West Pico or West Femto Maximum Sensitivity Chemioluminescent Substrate), and membranes were exposed to Kodak Biomax films.

4.6 Metabolic studies

Pulse experiments with [^{3}H]-DHSph. T98G and TMZ-R cells were plated on 35 mm dishes in cell culture medium at 3 x 10^5 cells/dish. Cells were fed with 1 μM [^{3}H]-DHSph (60 Ci/mmol) in growth medium for 2 hours and then incubated with 0.5 mM TMZ. After 16 and 38 hours, adherent and floating cells were collected and submitted to lipid extraction, partitioning and methanolysis, as described below.

Pulse experiments with [^{3}H]-Sph. U87-MG and GSCs cells were plated on 35 mm dishes in cell culture medium at 5 x 10^5 cells/dish. At the time of the experiment, the medium was gently removed and cells were then pulsed with 20 nM [^{3}H]-Sph (0.4 μCi/ml), for the indicated periods of time, in presence of growth medium containing 10 mM sodium glycerophosphate, 5 mM sodium fluoride, 0.5 mM deoxipyridoxine, and

0.5% BSA or 10% FCS, to trap released S1P [253]. At the end of the pulse cells and media were collected and processed as described below.

Extraction and partitioning of intracellular lipids

At the end of the pulse period, cells were harvested and total lipids were extracted at 4°C with chloroform/methanol 2:1 (v/v) as previously reported [254]. In particular, after mixing, samples were centrifuged at 10000 x g for 15 minutes at 4°C. The supernatant was collected and the precipitated was re-extracted with 200 µl chloroform/methanol 2:1 (v/v), mixed and centrifuged. The second supernatant obtained was added to the first one. The two supernatants combined represent the total lipid extract (ELT). An aliquot of the ELT was counted for radioactivity by liquid scintillation. Pellets obtained after lipid extraction, containing cellular proteins, were dried a under nitrogen stream and digested over night with 50 µl 1N NaOH at RT. Digestion was stopped with 450 µl distilled water. Aliquots were analyzed for protein content with the Lowry based assay.

ELT was then partitioned adding 260 µl of 0.1 M NH_4OH. After vigorous and repeated shaking, phases were separated by centrifugation at 8500 x g for 5 minutes at 4°C. At the end of the centrifugation a biphasic solution was obtained, composed of an upper aqueous phase (FA), containing the more polar intracellular lipids (S1P and gangliosides) and a lower organic phase (FO), containing the more apolar intracellular lipids (Cer, GlcCer , LacCer, Sph, and SM). Both FA and FO were evaporated under a nitrogen stream. FA was dissolved in chloroform/methanol/concentrated HCl 100:100:1 (v/v/v), counted for radioactivity by liquid scintillation and then analysed by HPTLC (as described below). Meanwhile FO was submitted to mild alkaline methanolysis.

Mild alkaline methanolysis of the FO

Since the FO obtained by ELT contains not only tritated sphingolipidic metabolites but also glycerophospholipids, it was submitted to a mild alkaline methanolysis to remove glycerophospholipids [254]. To this end, FO previously dried under nitrogen stream, was dissolved in 50 µl chloroform and then 50 µl of 0.2 N KOH in methanol were added. Samples were mixed and incubated 1 hour at 37°C under constant stirring. The reaction was stopped by neutralization with 60 µl of 0.2 N HCl in methanol. In order to reach the final chloroform/methanol ratio 2:1 (v/v), 90 µl methanol and 350 µl chloroform were added. After mixing, phases were separated by the addition of 95 µl water and centrifugation at 8500 x g for 5 minutes at 4°C. The upper aqueous phase was removed and the lower organic phase was dried under a nitrogen stream, re-

suspended in 300 µl chloroform/methanol 2:1 (v/v), counted for radioactivity and analysed by HPTLC (as described below).

Extraction and partitioning of extracellular S1P

Extracellular S1P was extracted from pulse medium and purified, as described elsewhere [174,175]. In particular, a two-step partitioning was performed, at first in alkaline conditions and then a back extraction of the aqueous phase obtained was carried out in acidic conditions according to Yatomi et al. (1995) [255] and Edsall and Spiegel (1999) [256] with some modifications. Briefly, 400 µl of medium were added to 750 µl of cold chloroform/methanol/concentrated HCl 100:200:1 (v/v/v). Samples were mixed at 4°C and phases were separated by the addition of 500 µl of chloroform and 130 µl of 3.5 N NH_4OH. After centrifugation at 10000 x g for 5 minutes at 4°C, the upper aqueous phase, containing volatile metabolites and extracellular S1P, was further partitioned adding 750 µl of chloroform and 40µl of 37% HCl. After mixing and centrifugation as above, the acidic organic phase obtained, containing extracellular S1P, was evaporated under nitrogen stream, resuspended in choloform/methanol/concentrated HCl 100:100:1 (v/v/v), and analysed by HPTLC. It has been previously demonstrated that in this condition about 90% of S1P is recovered [174]. Meanwhile the alkaline acqueous phase obtained was analyzed to determine [^{3}H]-S1P degradation.

Determination of S1P degradation

The tritiated water produced during [^{3}H]-S1P degradation was determined purifying, by fractional distillation, the alkaline acqueous phase obtained during the partitioning of the culture medium [254]. An aliquot of this aqueous phase was diluted in water and submitted to distillation. The fraction distilling at 100°C was collected and counted for radioactivity by liquid scintillation. It has been previously demonstrated that no loss of tritium by evaporation occurred under the experimental conditions used [174].

Separation and identification of [^{3}H]-sphingolipids by HPTLC

After extraction and partitioning, the fractions containing [^{3}H]-sphingolipids were submitted to high performance thin layer chromatography (HPTLC) on silica gel plates, using an opportune solvent system in a chromatographic chamber.

Sphingolipids contained in the cellular FO of cells pulsed with [^{3}H]-DHSph or [^{3}H]-Sph, were separated using as solvent system chloroform/methanol/acetic acid (190:9:1, v/v/v) or chloroform/methanol/water (110:40:6,

v/v/v), respectively; a standard mix composed of tritiated Cer, GlcCer , LacCer, Sph, and SM was chromatographed on the same plate and used as internal standard.

The fractions containing cellular and extracellular S1P were separated using n-butanol/acetic acid/water (3:1:1, v/v/v) as solvent system. Standard [^{3}H]-S1P was chromatographed on the same plate and used as internal standard.

At the end of the chromatography, HPTLC plates were dried and submitted to digital autoradiography (Beta-Imager 2000, Biospace, Paris, FR). Sphingolipids were quantified by the beta vision analysis software (Biospace, Paris, FR).

4.7 Sphingosine kinase activity

SKs activity was evaluated both intracellularly and in cell-conditioned medium in order to assess whether GSCs released these enzymes in the extracellular milieu. To this end, U-SC (5 x 10^5 cells/dish) were incubated at 37°C in fresh culture medium for different periods of time (30, 60 minutes and 24 hours). Subsequently cells and media were collected in order to evaluate SKs activity.

Cells were harvested in SK buffer (20 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 0.5 mM deoxypiridoxine, 15 mM NaF, 1 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 40 mM sodium glycerophosphate, 10% glycerol and protease inhibitors) and disrupted by freeze-thawing. Protein concentration was determined through the Lowry based method.

SKs activity was assayed using experimental conditions known to selectively favour SK1 or SK2 activity with minor modifications [152,154,167]. Briefly, the reaction mixture (final volume, 100 μL) contained: cell homogenate (15-30 μg), 50 μM D-erytrhro-sphingosine as BSA-complex, 1 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$ and SK buffer supplemented with either 0.5% Triton X 100 to asses SK1 activity, or 200 mM KCl to asses SK2 activity. The mixture was incubated at 37°C for 30 minutes.

Media were collected and concentrated by centrifugation at 5110 x g for 1 hour at 4°C. SKs activity was assayed preparing the reaction mixture (final volume, 100 μL) containing: concentrated medium (25-50μl), 25 μM D-erytrhro-sphingosine as BSA-complex, 0.5 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$ and SK buffer supplemented with either 0.5% Triton X100 or 200 mM KCl in order to asses SK1 or SK2 activity respectively. The mixture was incubated at 37°C for 30 minutes.

Both cells and media reactions were terminated and lipids were extracted, as previously described [174,239]. In particular reactions were stopped by the addition of 10 μL 1 M HCl followed by 400 μL chloroform/methanol/HCl (100:200:1, v/v/v). After mixing, 120 μL of chloroform and 120 μL of 2 M KCl were added and phases separated by centrifugation at 1000 x g for 10 minutes at RT. A biphasic solution was obtained, composed of an upper aqueous phase (FA) rich in labeled ATP and a lower organic phase (FO), containing labelled lipids. The FO was recovered and transferred into a test tube, evaporated to dryness under a stream of nitrogen and then dissolved in chloroform/methanol/concentrated HCl 100:100:1 (v/v/v). Labelled lipids in the organic phase were then resolved by HPTLC on silica gel plates using 1-butanol/methanol/acetic acid/water 8:2:1:2 (v/v/v/v) as solvent system. At the end of the chromatography, HPTLC plates were dried and labelled lipids visualized using autoradiography by overnight exposure to Kodak Biomax films. The radioactive spots corresponding to S1P were scraped from the plates and counted for radioactivity by liquid scintillation. Background values were determined in negative controls in which sphingosine was not added to the reaction mixture.

4.8 Protein assays

The determination of the protein content was performed using the Bradford/Comassie [257] method or the Lowry assay [258]. In both cases bovine serum albumin (BSA) was used as standard and a calibration curve was set up using increasing amounts of BSA. The protein content was obtained by spectrophotometric reading at 595 nm for the Bradford method and 750nm for the Lowry assay.

4.9 Statistical analysis

Results are expressed as means ± SD or SEM for at least three independent experiments. The statistical significance of the data was determined by the Student's t-test. Significant differences were accepted at least $p < 0.05$. Data were analyzed using StatMate software, version 4.0 (GraphPad).

RESULTS

5.1 Effect of TMZ on T98G cell viability

To examine the antitumour effect of TMZ on malignant glioma cells, we treated T98G cells with TMZ at concentrations ranging from 0.1 mM to 1.5 mM in complete medium. We found that this alkylating agent inhibits cell viability in a dose-dependent manner, and this effect increases with prolonged drug exposure (data not shown). After 72 hours of treatment with TMZ, the drug sensitivity parameters D_{37} and LD_{10} (indicating the TMZ doses required to reduce cell viability to 37% and 10% respectively [259]) were calculated. In particular, in T98G glioblastoma cells D_{37} was 285 μM and LD_{10} was 814 μM (Table 1).

As a model of drug-induced resistance in glioma cells, we used the TMZ-resistant (TMZ-R) variant previously obtained in our laboratory from T98G cells by selection with increasing drug concentrations. As shown in Table 1, the resistance parameters D_{37} and LD_{10} were increased in the TMZ-R variant, the corresponding resistance factors being more than five-fold higher than those of the parental T98G [252].

		TMZ	
Cell type		D_{37}	LD_{10}
T98G	mM	0.28	0.81
TMZ-R[a]	mM	1.74	> 4.10
	Resistance factor	6.10	>5.00

[a]Selected at 1.5 mmol/L of TMZ.

Table 1. TMZ cytotoxicity in T98G cells and T98G TMZ-resistant variant. T98G and TMZ-resistant cells were exposed to increasing doses of TMZ (0.1-1.5 mM). Cell viability was evaluated after 72 hours by MTT assay. The drug sensitivity parameters D_{37}, LD_{10} and the corresponding resistance factor are shown.

5.2 Expression of the TMZ-resistance marker MGMT in T98G and TMZ-resistant cells.

One of the best characterized mechanisms of resistance to the alkylating agent TMZ is the increased expression of the DNA repair protein MGMT [37], which is able to remove the methyl groups added to the DNA by alkylating agents. Therefore, the role of this enzyme in TMZ sensitivity was evaluated. To this purpose, MGMT protein level was assessed both in T98G and TMZ-R cells by Western blot analyses. As

shown in Figure 13, our results demonstrated that MGMT expression levels were markedly higher in TMZ-R than in T98G cells, indicating that MGMT has a role in TMZ-resistance in this cell line.

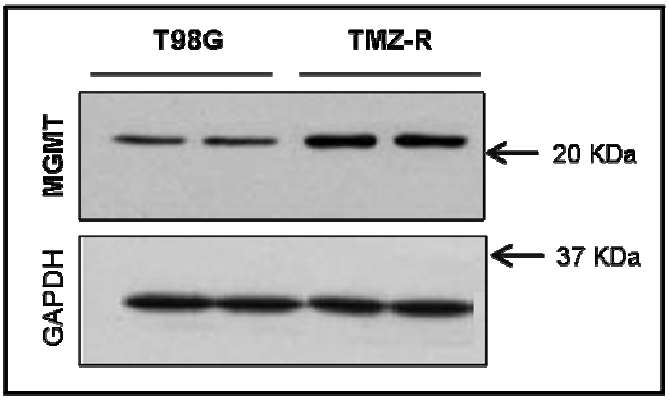

Figure 13. Expression of MGMT protein. T98G and TMZ-resistant cells were lysed and aliquots containing 20 µg of proteins were analyzed by immunoblotting assays with anti-MGMT and anti-GAPDH antibodies. The immunoblottings shown are representative of one out of three identical experiments performed.

5.3 Effect of TMZ on the regulation of ceramide levels in T98G cells

An increasing amount of evidence indicates that ceramide plays an important role as a tumour suppressor lipid, is able to induce antiproliferative and apoptotic responses, and acts as a major player in the mechanism of action of many chemotherapeutic drugs [107,260,261]. On this premise, we investigated whether TMZ treatment was involved in ceramide formation. To this purpose, we labelled cells for 2 hours with [^{3}H]-DHSph, as ceramide precursor. We then evaluated ceramide content after 16 hours of TMZ treatment, when no significant variation in the number of adherent cells was measurable, and later on, at 38 hours, when adherent cells were reduced and numerous cells floating in the medium were evident after treatment (Fig. 14A and 14B).

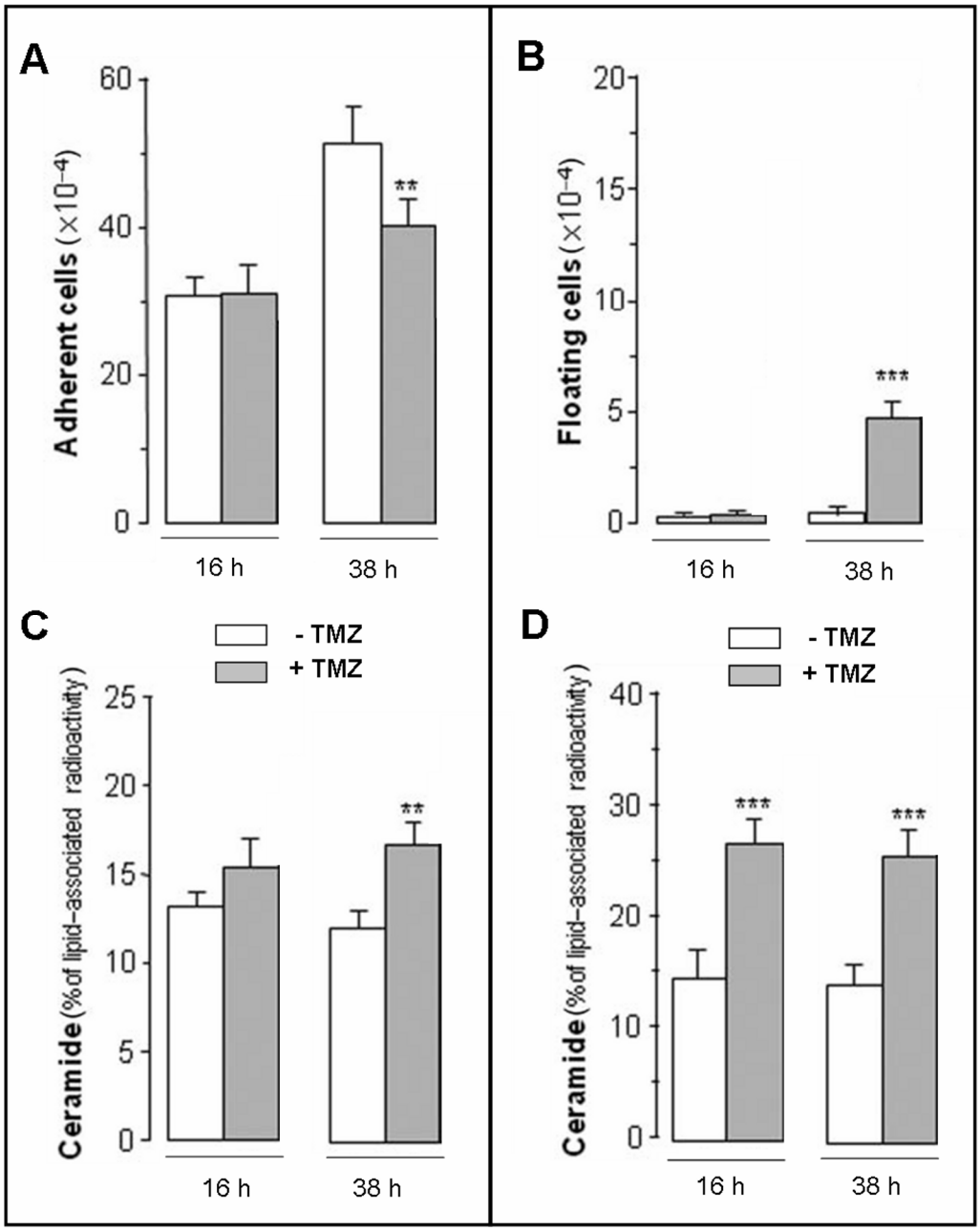

Figure 14. TMZ induces cell death and increases ceramide level in T98G cells. Cells were incubated with 1 μM [^{3}H]-DHSph (60 Ci/mmol) for 2 hours, and then in the absence (-TMZ) or presence of 0.5 mM TMZ (+TMZ) for 16 or 38 hours. At the end, adherent **(A)** and floating cells **(B)** were counted and resulted trypan blue negative and positive, respectively. At the end of incubation, both adherent **(C)** and floating cells **(D)** were also processed and analyzed to evaluate the radioactivity incorporated into ceramide, as described in "Materials and methods". Ceramide is expressed as the percentage of radioactivity incorporated into the lipid extract. Data are the mean ± SD of three independent experiments. Asterisks represent statistical significance determined by Student's t test (** $p < 0.01$, ***$p < 0.001$ vs un-treated cells).

Trypan blue analyses revealed that the large majority (>98%) of adherent cells were vital, and that floating ones were trypan blue positive, i.e. dead. We therefore analysed both adherent and floating cells (representing vital and dead cells, respectively) for their ceramide content. At both times of treatment, the cell-incorporated radioactivity (as sum of adherent and floating cell-associated radioactivity) was similar in control and TMZ-treated cells (data not shown), thus indicating that the drug treatment did not affect DHSph incorporation into the cells. The results of the ceramide content in adherent cells revealed a time-dependent increase in TMZ-treated cells (Fig. 14C). Interestingly, the ceramide level in drug-treated floating cells was markedly higher (about two-fold) than in control ones, independently of the time (Fig. 14D).

Notably, and in contrast to what it occurs in T98G, in TMZ-R cells, no significant variation in the ceramide level was found after treatment with TMZ (up to 1 mM) (data not shown) [252].

5.4 Effect of ceramide on T98G cell viability

Prompted by these findings, we evaluated the effect of ceramide on cell fate. To this purpose, T98G cells were exposed for 24 hours to different concentrations of cell-permeable ceramide analogues and cell death was determined. As shown in Figure 15A, exposure of T98G to increasing doses of C2-ceramide and C6-ceramide resulted in a dose-dependent effect on cell death. Of relevance, as shown in Figure 15B, this cytotoxic effect was mimicked by a long-chain ceramide derived from bovine brain sphingomyelin, containing 18:0, 24:1, and 24:0 as major fatty acid species [262]. As expected on the basis of their cell permeability, the potency of the cytotoxic effect of the different ceramides was inversely related to the length of their N-acyl chain (Fig. 15B) [252].

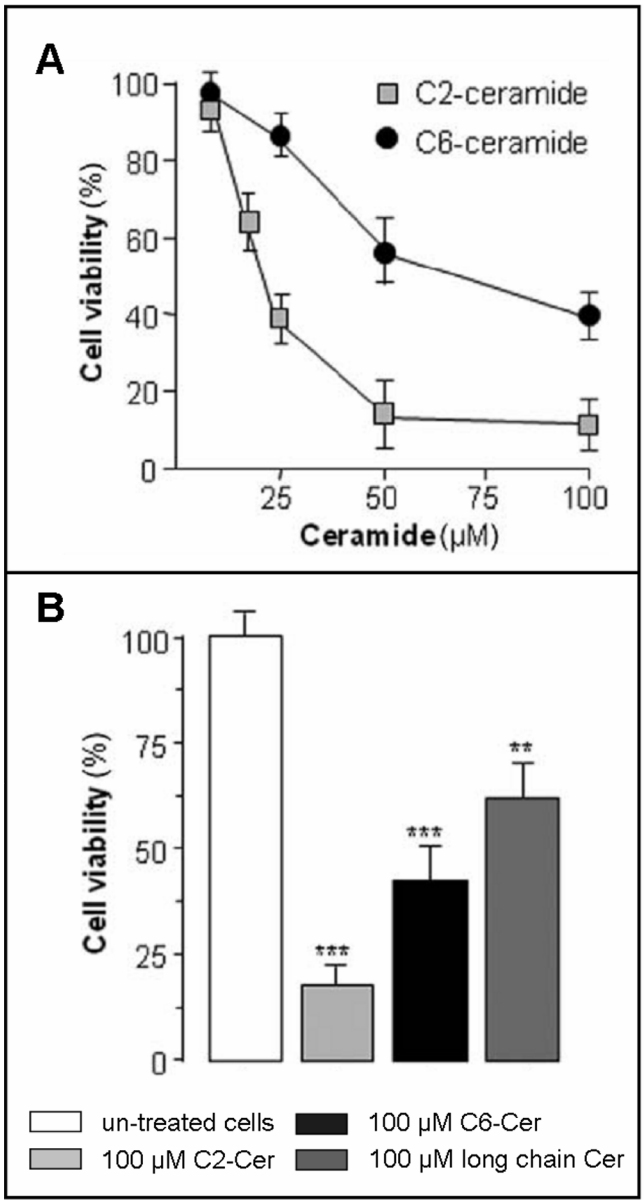

Figure 15. Ceramides induce cell toxicity in T98G glioblastoma cells. T98G cells were exposed to different doses of C2- and C6-ceramide **(A)**, and 100 μM C2-ceramide, C6-ceramide or long-chain ceramides **(B)**. After 24 hours, cell viability was assessed by MTT assay. Results are expressed as percentage of cell viability with respect to un-treated cells (regarded as 100%). Data are mean ± SD of three-five independent experiments. Asterisks represent statistical significance determined by Student's t test (** $p < 0.01$, ***$p < 0.001$ vs un-treated cells).

5.5 Isolation and characterization of GSCs

Stimulated by these results, we decided to evaluate the role of sphingolipids also in GSCs, a cell subpopulation within the tumour involved in the aberrant expansion and therapy resistance properties of glioblastomas [50,51]. To this purpose we isolated GSCs from the human U87-MG glioblastoma multiforme cell line, known to be particularly enriched with cancer stem cells [263].

GSCs were isolated from U87-MG using a selective growth medium, which promotes the proliferation of the stem cell component only (U-SC). The isolation process usually lasts one month, during which, in selective serum-free conditions, differentiating/differentiated cells rapidly die, while the stem cell component proliferate forming compact cell clusters floating in suspension, called “neurospheres”. Importantly, only a fraction of the initial neurospheres, ranging from 10 to 50% of the total cells, retains stem cell features, while the remainder of the cells undergo spontaneous differentiation [250]. In fact, an initial neurosphere is a mixture of stem cells, differentiating progenitors, and even terminally differentiated glioblastoma cells. This is the reason why, in order to obtain in the progressive enrichment and stable expansion of U-SC in culture, during the isolation process every week neurospheres were subcultured by harvesting, followed by dissociation and re-plating under the same growth conditions.

Images acquired by phase-contrast microscopy (Fig. 16A), showed obvious morphological differences between the two cell types. U87-MG cells grew adherent to the surface of the growth support, with a fibroblastoid-like morphology characterized by the formation of numerous cytoplasmic extensions (Fig. 16A). Conversely, U-SC grew in suspension in the culture medium forming typical neurosphere structures (Fig. 16A), composed of 100-200 cells.

It has been reported that brain tumour-derived cancer stem cells express high levels of the membrane glycoprotein CD133 and the cytoskeleton protein nestin [54,55]. In order to assess if the population of cells obtained by selective isolation was actually enriched in stem cells, the expression of stemness markers was evaluated by a Real-Time PCR assay in both U87-MG and U-SC cells. Our results (Fig. 16B) showed that U-SC presented a 5.3- and 9.5-fold increase in CD133 and nestin expression respectively, compared to U87-MG. Thus U-SC we obtained by selective isolation were characterized by stem cell typical morphology and by increased expression of stemness markers, demonstrating that U-SC are representative of GSCs population.

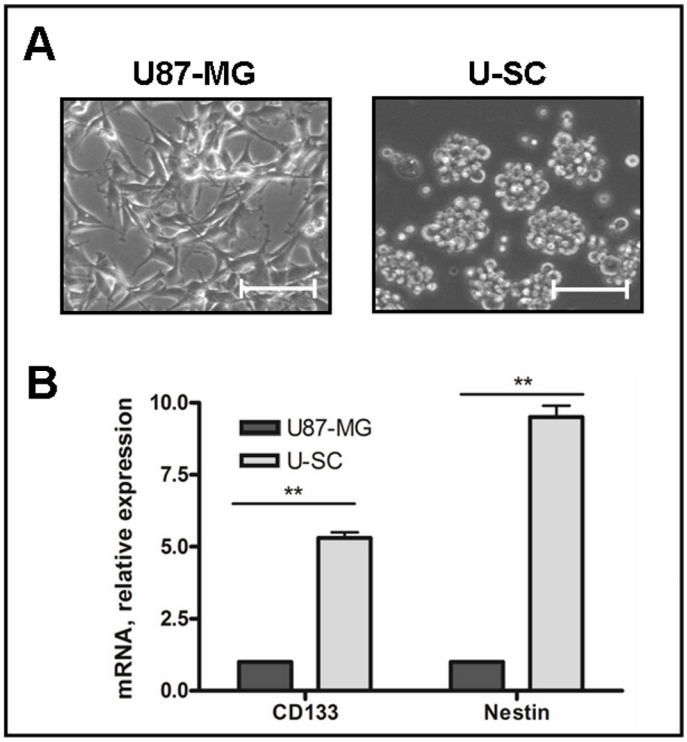

Figure 16. Characterization of GSCs derived from U87-MG. **(A)** Representative images of U87-MG and U-SC morphology. Images were viewed on a contrast phase microscope and digital images were acquired (magnification 10X, Scale bar 100 µm). **(B)** Relative expression of CD133 and nestin, assessed by Real-Time PCR. Data were normalized to GAPDH expression (used as endogenous control) and U87-MG were used as calibrator. Results are shown as fold-change relative to marker expression in U87-MG, sets arbitrarily to 1. Values are mean ± SEM of three independent experiments. Asterisks represent statistical significance determined by Student's t test (** $p < 0.01$ U-SC cells vs U87-MG cells).

As additional *in vitro* model for GSCs, we exploited L0627, previously isolated from a post-surgery specimen of primary human glioblastoma multiforme. These GSCs reproduce the genotypic and phenotypic characteristics of glioblastomas more faithfully than standard glioma cell lines [48,264]. L0627 grew in suspension in the culture medium and efficiently formed neurospheres (Fig. 17A). Moreover, these cells were characterized by the high expression levels of putative cancer stem cell markers (such as CD133 and the adhesion molecule CD15) (Fig. 17B) and by self-renewal, multipotency and tumorigenicity, as previously described [48,265]. In particular, these cells, similarly to the normal neural stem cell counterpart, emerged as multipotent, thus able to simultaneously generate cells displaying antigenic reactivity for neuronal, astroglial and oligodendroglial markers in culture. More importantly, these cells were described as tumour-founding

cells, able to establish tumours that closely resemble the main histologic, cytologic and architectural features of the human disease, even when challenged through serial transplantation.

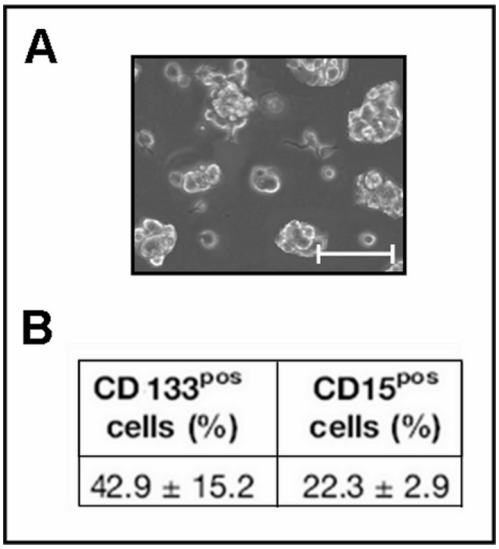

Figure 17. Characterization of GSCs derived from primary culture of human glioblastoma multiforme. **(A)** Representative image of L0627 morphology viewed on a contrast phase microscope (magnification 10X, Scale bar 100 μm). **(B)** CD133 and CD15 expression was assessed by flow cytometry analyses.

5.6 Effect of TMZ on U87-MG and GSCs viability

In order to analyze cell survival properties, increasing concentrations of the alkylating agent TMZ (ranging from 25 to 200 μM) were used to treat U87-MG, U-SC and L0627. After 48 hours of treatment the cytotoxic effect on cells was evaluated. Images of cells obtained by phase contrast microscopy show the morphologic effects of treatment with 100 μM TMZ for 48 hours: many of U87-MG cells were found dead in suspension, while those still adherent to the growth surface were strongly suffering, since characterized by a decrease of cell size and by the loss of normal fibroblastoid-like morphology (Fig. 18A); in contrast, U-SC and L0627 (Fig. 18A-B), under the same experimental conditions, maintained unchanged their morphological characteristics with the typical formation of neurospheres.

These morphologic data are supported by the analysis of cell viability by MTT assay. The results obtained show that in U87-MG cells the treatment with TMZ for 48 hours decreased cell viability in a dose-dependent manner. Indeed the treatment with TMZ 100 μM was associated with a cell survival of 60%, meanwhile the dose 200 μM caused more than 50% of cell death (Fig. 18C). Conversely treatment with TMZ at all doses

considered did not cause significant alterations in U-SC vitality (Fig. 18C). Also L0627 viability did not significantly change after 48 hours of treatment with TMZ at doses 25-100 μM. The highest TMZ concentration (200 μM) was only partial effective on L0627, indeed about the 70% of cells were still alive (Fig. 18D). Altogether these results demonstrated that at doses that were cytotoxic in U87-MG cells, TMZ was uneffective on U87-derived GSCs viability. Moreover, TMZ had a limited effect on L0627 survival only at the highest dose administrated.

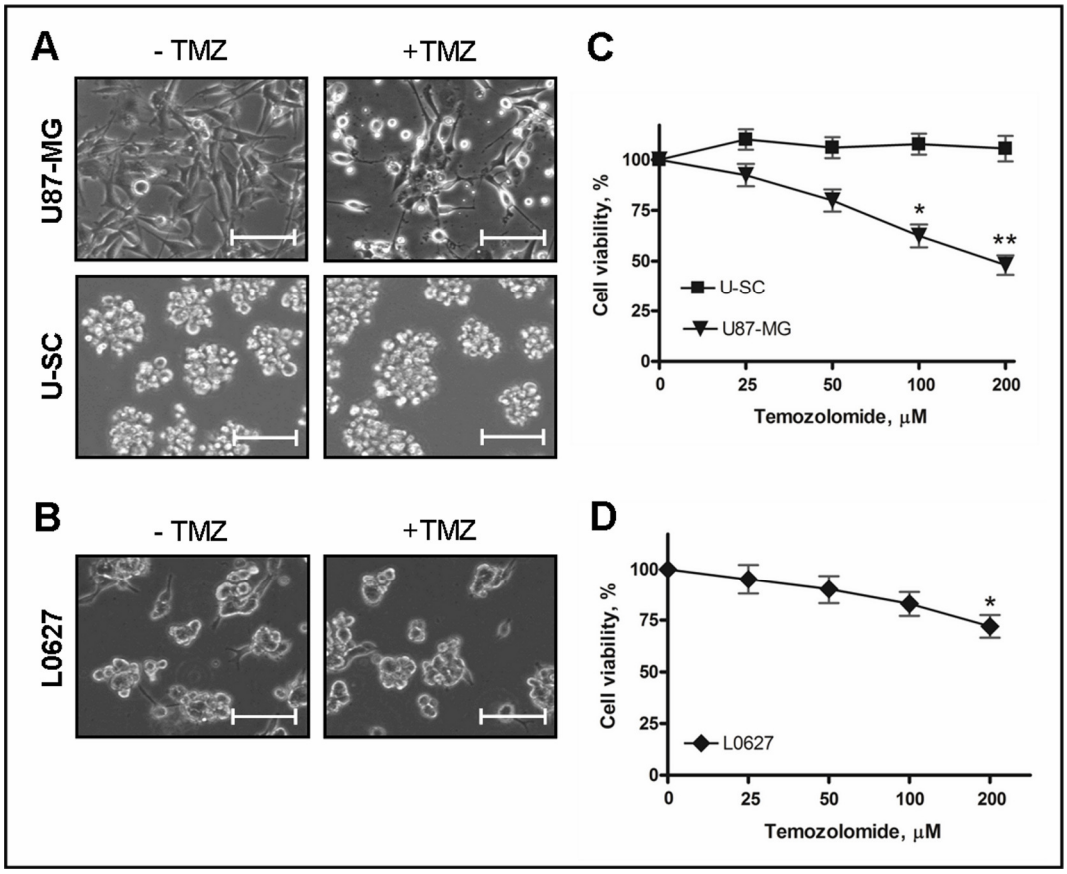

Figure 18. Effect of TMZ on U87-MG and GSCs viability. Representative images of **(A)** U87-MG, U-SC and **(B)** L0627 morphology after 48 hours of treatment with vehicle (-TMZ; 0.1% DMSO) or 100 μM TMZ (+TMZ). Images were viewed on a phase contrast microscope and digital images were acquired (magnification 10X, Scale bar 100 μm). U87-MG, U-SC **(C)** and L0627 **(D)** were exposed to different doses of TMZ (25-200 μM) or vehicle. Cell viability was assessed after 48 hours of treatment by MTT assay. Results are expressed as percentage of cell viability with respect to vehicle-treated cells (100%). Data are mean ± SEM of three independent experiments. Asterisks represent statistical significance determined by Student's t test (* $p < 0.05$ and ** $p < 0.01$ vs vehicle-treated cells).

5.7 Expression of the TMZ-resistance marker MGMT in U87-MG and GSCs

The DNA repair enzyme MGMT is expressed in most of human gliomas, its levels being directly related to tumour resistance to alkylating drugs [37]. Therefore immunoblotting assays were performed in U87-MG, U-SC and L0627 in order to evaluate MGMT protein levels. As shown in Figure 19, MGMT (21 kDa) was not expressed not only in U87-MG, but surprisingly also in U-SC and L0627.

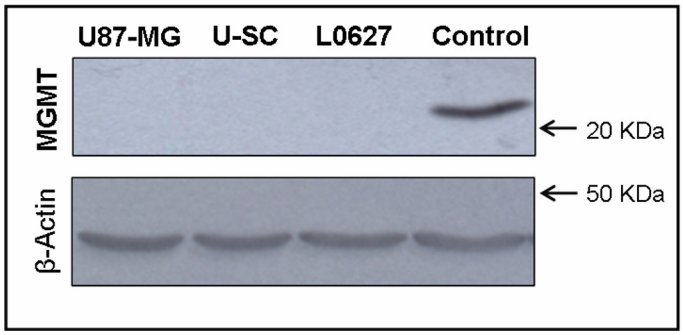

Figure 19. Expression of MGMT protein. U87-MG, U-SC and L0627 were lysed and aliquots containing 60 µg of proteins were analyzed by immunoblotting assays with anti-MGMT and anti-β-actin antibodies. Lysates of T98G glioblastoma cell line were used as positive control. The immunoblottings shown are representative of one out of three similar experiments.

5.8 Expression of SK in U87-MG and GSCs

Since data from our laboratory supported a role for S1P in TMZ resistance, we evaluated the expression of the two known isoforms of the enzyme sphingosine kinase (SK1 and SK2), responsible for S1P synthesis, in U87-MG, U-SC and L0627 cells. As shown in Figure 20, results obtained by immunoblotting assays demonstrated that U87-MG and the two GSCs models expressed both SK1 and SK2.

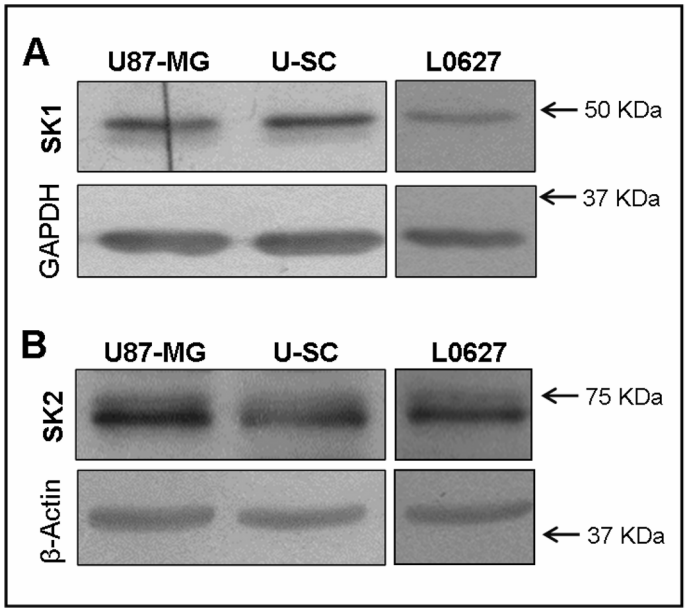

Figure 20. U87-MG, U-SC and L0627 express both SK1 and SK2. Cell lysate aliquots containing 40 µg of proteins were analyzed by immunoblotting with anti-SK1 and anti-GAPDH antibodies **(A)** or anti-SK2 and anti-β-actin antibodies **(B)**. The immunoblottings shown are representative of one out of three similar experiments.

5.9 S1P biosynthesis and fates in U87-MG and GSCs

In order to evaluate S1P production efficiency and its metabolic fate, short time pulse experiments were performed in U87-MG and both GSCs by administering to cells [^{3}H]-Sph, which is efficiently internalized by cells, as S1P precursor. As shown in Figure 21A, the incorporated radioactivity increased in a time-dependent manner and was similar in all cell models. Furthermore, residual intracellular [^{3}H]-Sph represented about only the 3% of the incorporated radioactivity, suggesting that all cells are able to rapidly metabolize sphingosine (Fig. 21A).

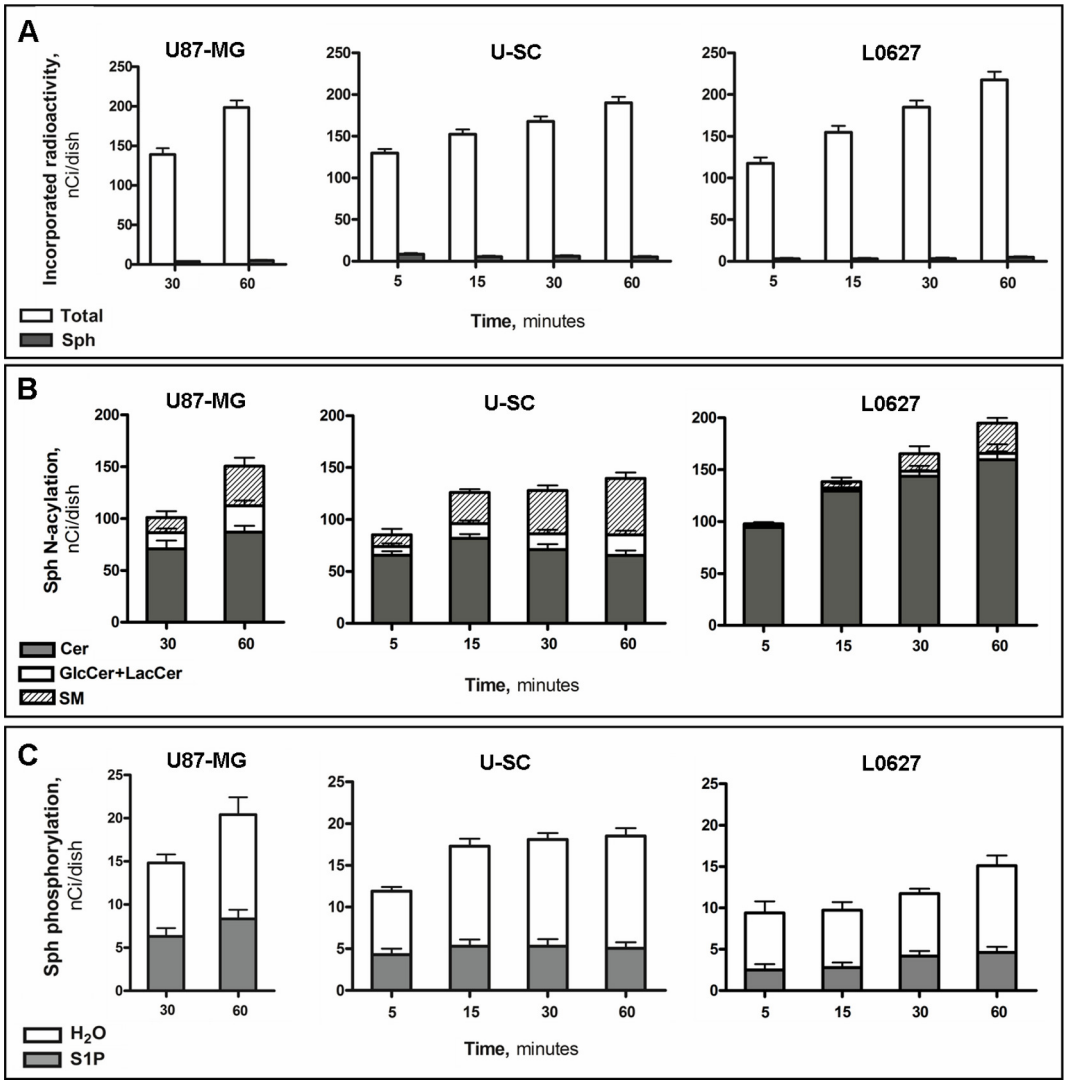

Figure 21. [^{3}H]-Sph metabolism in U87-MG and GSCs. U87-MG, U-SC and L0627 were pulsed with 20 nM [^{3}H]-Sph (0.4 µCi/ml) for the indicated periods of time. At the end of incubation, cells and media were processed and analyzed as described in "Materials and methods". In panel **A** incorporated radioactivity and intracellular unmetabolized [^{3}H]-Sph are shown. Panel **B** displays the radioactivity associated to the process of sphingosine N-acylation. Panel **C** shows the radioactivity associated to the process of sphingosine phosphorylation. All values are mean ± SD of at least three independent experiments.

Of note, in all cell models and at all investigated times, most of the incorporated [^{3}H]-Sph (about 80%) was used in the N-acylation process, that is in the production of ceramide and its use for the synthesis of complex sphingolipids (glycosphingolipids and SM). Figure 21B shows that in all cell models the radioactivity representing sphingosine N-acylation process increased in a time dependent manner. In particular, [^{3}H]-Cer represented the bulk of Sph N-acylation-associated radioactivity and increased over pulse time in U87-MG and L0627, whereas it showed no significant variations in U-SC. Furthermore all cells were characterized by

a time dependent increase in the levels of ceramide metabolism products: [^{3}H]-SM, [^{3}H]-GlcCer and [^{3}H]-LacCer. Of relevance, at all considered experimental times, [^{3}H]-Cer levels were found higher in L0627 than in U-SC; meanwhile, the radioactivity associated with complex sphingolipids was lower in L0627 than in U-SC, suggesting that in U-SC ceramide metabolism is faster and more efficient with respect to L0627.

We then focused on the sphingosine phosphorylation process which can be measured on the basis of S1P production and degradation. Figure 21C shows that in all cell models the radioactivity representing sphingosine phosphorylation increased over pulse time. [^{3}H]-S1P degradation was measured as tritiated water. In fact, since [^{3}H]-S1P carries the radioactivity at the level of the C3, its degradation by S1P lyase produces unlabeled ethanolamine phosphate and [^{3}H]-hexadecenal. With further dehydrogenation, the radioactive fatty aldehyde loses tritium with the consequent formation of tritiated water [266]. Interestingly, at all investigated times, tritiated water represented the bulk of Sph phosphorylation-associated radioactivity and raised with the increase of the pulse times (Fig. 21C) suggesting that U87-MG and both GSCs are able to efficiently synthesize and degrade S1P. Moreover, the radioactivity associated to S1P production had a similar trend in all cell models and represented about the 35% of the sphingosine phosphorylation process at all times considered (Fig. 21C).

Since astrocytes, cerebellar granule cells and glioma cells are able to release S1P in the extracellular milieu [174,175,237,239], we analyzed both cells and culture media in order to evaluate [^{3}H]-S1P distribution in U87-MG and GSCs.

Concerning U87-MG, the radioactivity associated with intracellular S1P increased in a time-dependent manner, as shown in Figure 22A. Additionally, a small amount of S1P, corresponding to the 3% of the radioactivity associated to sphingosine phosphorylation, was detected extracellularly at both 30 and 60 minutes (Fig. 22B).

Data obtained in U-SC show that the radioactivity associated with intracellular S1P decreased over pulse time (Fig. 22A). Meanwhile, in L0627, intracellular S1P radioactivity increased at 30 minutes of pulse and remained constant at 60 minutes (Fig. 22A). Furthermore, in both GSCs models the radioactivity associated with extracellular S1P increased with the pulse time (Fig. 22B). Intriguingly, in U-SC extracellular [^{3}H]-S1P levels triplicated at 60 minutes with respect to 5 minutes of pulse. Similarly, in L0627 the radioactivity associated to extracellular S1P increased more than twice at 60 minutes with respect to 5 minutes of pulse. Of note, at all experimental times considered, tritiated water and [^{3}H]-S1P levels were lower in L0627 than in U-SC, suggesting that U-SC are able to phosphorylate Sph more quickly and efficiently than L0627.

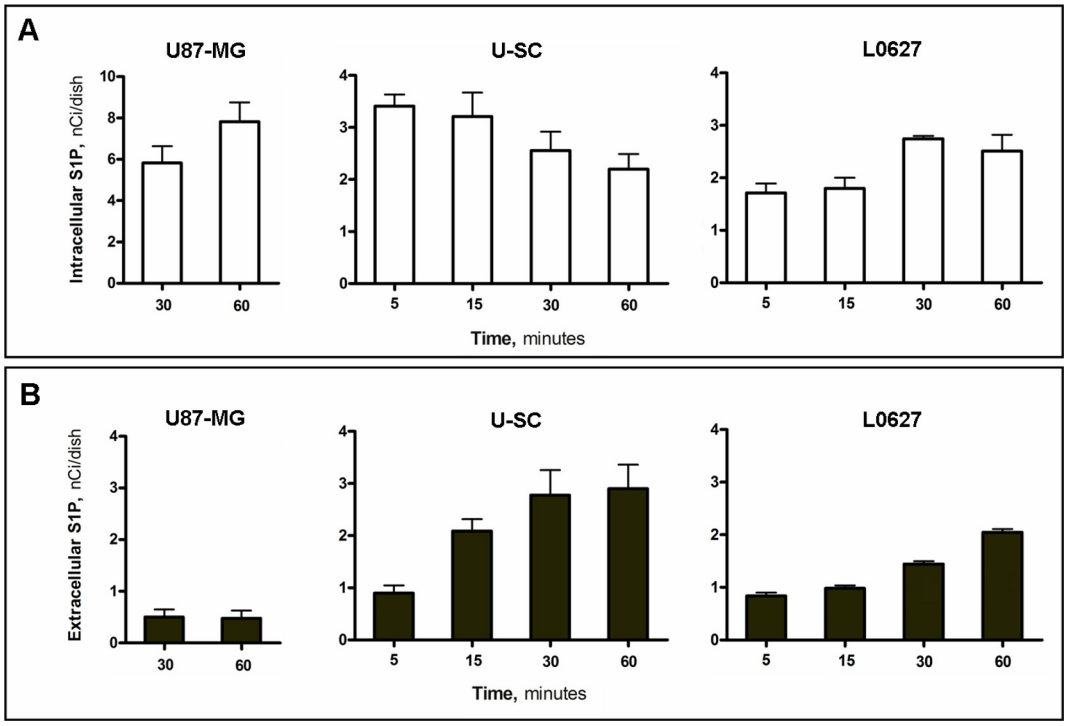

Figure 22. Intracellular and extracellular [^{3}H]-S1P in U87-MG and GSCs. U87-MG, U-SC and L0627 were pulsed with 20 nM [^{3}H]-Sph (0.4 µCi/ml) for the indicated periods of time. At the end of incubation, cells and media were processed and analyzed as described in "Materials and methods". The radioactivity incorporated into intracellular (panel **A**) and extracellular S1P (panel **B**) is shown. All data are expressed as nCi/dish. All values are mean ± SD of at least three independent experiments.

Comparing results obtained in U87-MG and GSCs, we showed that in the former the radioactivity associated with intracellular S1P was at least 2-fold higher than in the latter. In contrast, the radioactivity associated with extracellular S1P in U87-MG was from 3 to 6 times lower with respect to GSCs. These results demonstrated that, in conditions of similar radioactivity incorporation, the extracellular/intracellular S1P ratio was much higher in GSCs than in U87-MG (Fig. 23A). In particular, both GSCs showed a ratio of about 1 which was at least 10 times higher than that in U87-MG.

Importantly, S1P detection in GSCs medium was not accompanied by LDH release (data not shown), suggesting that the presence of extracellular S1P was not a consequence of cell membrane rupture.

Furthermore, these differences in extracelluar [^{3}H]-S1P levels between U87-MG and GSCs resulted also in a ceramide-extracellular S1P ratio lower in GSCs than in U87-MG. In particular this ratio was from 6 to 8 times lower in U-SC with respect to U87-MG (Fig. 23B). Moreover, despite in L0627 [^{3}H]-Cer intracellular levels were about 2 times higher than in U87-MG, the relevant amount of [^{3}H]-S1P found in L0627 medium led to a ceramide-extracellular S1P ratio which was significantly lower (more than 1.5 fold) in L0627 than in U87-MG

(Fig. 23B). According to the "sphingolipid rheostat" model [111], we hypothesized that this different ratio could promote GSC survival observed after TMZ treatment. Interestingly, this ratio is higher in L0627 with respect to U-SC, leading us to speculate that this could be the reason why the highest dose of TMZ used (200μM) had a partial effect on L0627 and no effect on U-SC viability.

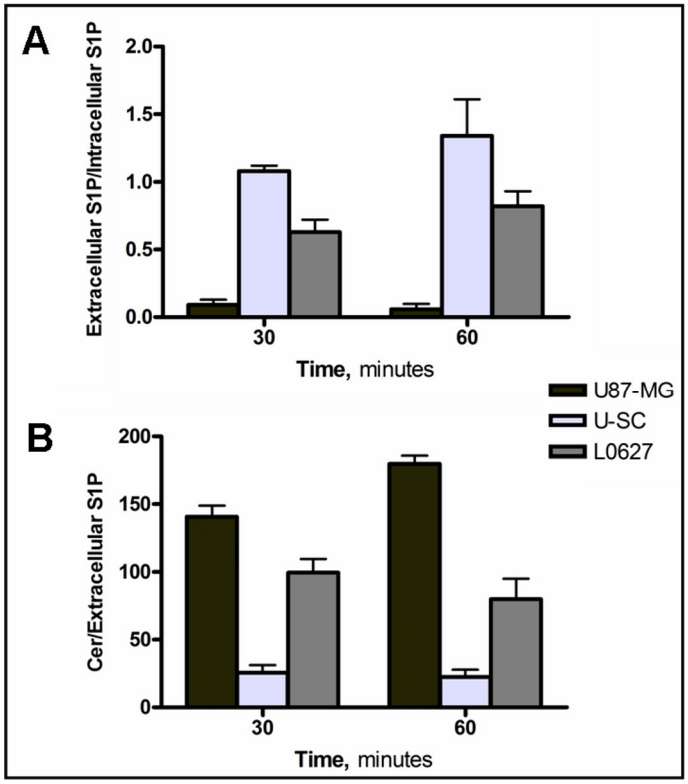

Figure 23. Extracellular S1P-intracellular S1P and Cer-Extracellular S1P ratios in U87-MG and GSCs. U87-MG and both GSCs were pulsed with 20 nM [^{3}H]-Sph (0.4 μCi/ml) for 30 or 60 minutes. At the end of incubation, cells and media were processed and analyzed as described in "Materials and methods". Histograms represent the ratio between extracellular and intracellular S1P-associated radioactivity **(A)** and the ratio between ceramide and extracellular S1P-associated radioactivity **(B)**. Data are the mean ± SD of at least three independent experiments.

5.10 Analysis of SK activity in U-SC and in their culture medium

We then hypothesized that the high amount of S1P retrieved in GSCs medium could be consequent to sphingosine kinase secretion in the extracellular milieu, as it occurs in some cell types [167-169]. On this base, we performed enzyme activity assays of the two sphingosine kinase recognized isoforms (SK1-SK2) on U-SC and their medium, using experimental conditions able to detect also low SK activity. Our results demonstrated the presence of the two SK isoforms inside the cells and the absence of these two enzymes in the culture medium (Fig. 24). These data excluded our hypothesis, suggesting that a very efficient transport mechanism is involved in S1P export from GSCs.

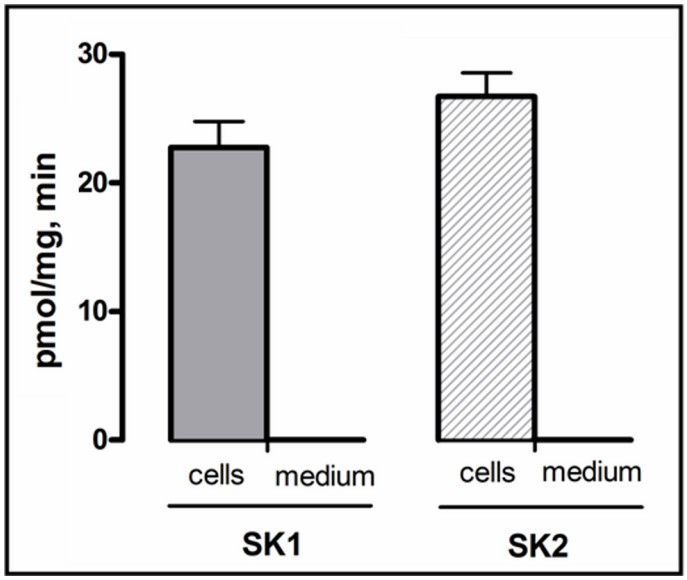

Figure 24. SK activity in U-SC and in their culture medium. U-SC were incubated at 37°C in fresh culture medium for 24 hours. Subsequently cells and media were collected in order to evaluate SKs activity as described in "Materials and methods". Enzyme activity was measured using sphingosine and [γ-^{32}P]ATP as substrates, under buffer conditions favouring SK1 or SK2 activity. Data are the mean ± SD of at least three independent experiments.

5.11 Expression of ABCG2, ABCC1 and ABCA1 membrane transporters in U87-MG and GSCs

The mechanism of S1P transport across the plasma membrane is still unclear. Increasing evidence supports that different subfamilies of ATP binding cassette (ABC) transporters contribute to sphingolipid passage across the membrane. In particular ABCG2, ABCA1 and ABCC1 were found involved in the S1P export to the extracellular milieu [173,178,180]. On this base we then analyzed, in U87-MG and both GSCs, the expression of these three ABC transporters.

Data obtained through immunoblotting assays demonstrated that ABCG2 is expressed in U87-MG cells, meanwhile in U-SC it is expressed at very low levels, at the limit of method-sensitivity, and it is was not found to be expressed in L0627 (Fig. 25A). Furthermore, no band for ABCC1 expression was detected either in U87-MG or in GSCs (Fig. 25C). Only ABCA1 resulted present in GSCs, and it appeared to be much more expressed in U87-MG than in both GSCs (Fig. 25B).

These findings stimulated us to analyze the possible involvement of this transporter in the extracellular release of S1P by GSCs. Further analyses revealed that ABCA1 inhibition, through the sulfonylurea derivative glyburide, caused no variations in L0627 ability to release S1P (data not shown).

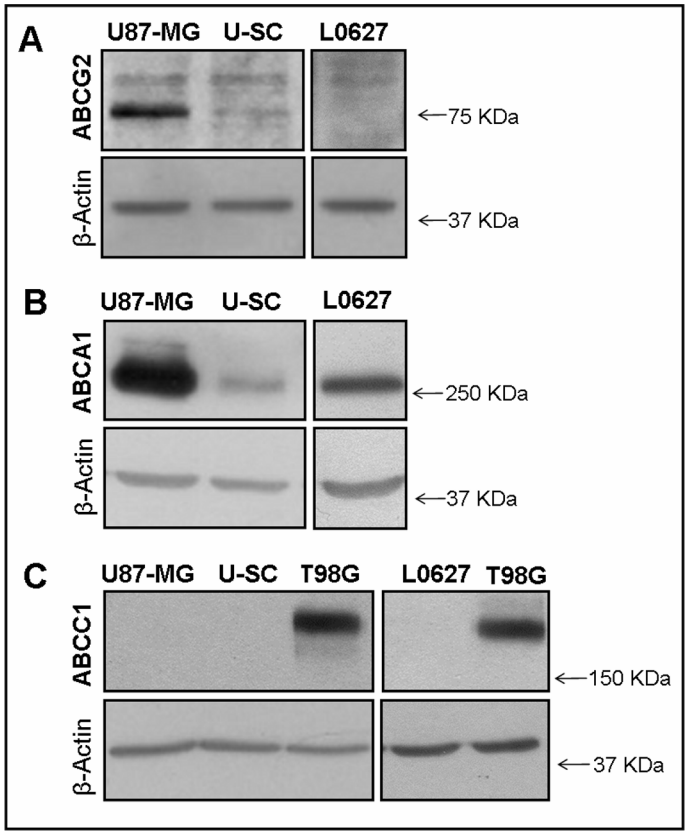

Figure 25. Expression of ABCG2, ABCC1 and ABCA1 membrane transporters in U87-MG and GSCs. Cell lysate aliquots containing 40 µg of proteins were analyzed by immunoblotting with anti-ABCG2 **(A)**, anti-ABCA1 **(B)** or anti-ABCC1 **(C)** antibodies. β-actin levels were used as protein-loading control. Lysates of T98G glioblastoma cell line were used as positive control for ABCC1 expression.The immunoblottings shown are representative of one out of three identical experiments performed.

5.12 S1P biosynthesis and fates in L0627 treated with TMZ

The evidence that GSCs are resistant to TMZ and their ability to release S1P in the extracellular microenvironment, stimulated us to analyze the effect of TMZ treatment on S1P biosynthesis and fates in GSCs. To this purpose L0627 were incubated with 100 μM TMZ for 24 hours and then pulsed with tritiated sphingosine for 1 hour. Total incorporated radioactivity was found similar in un-treated and treated cells (217 nCi/dish and 207 nCi/dish, respectively). Sphingosine N-acylation and phosphorylation processes had a similar trend, with no significant variations, in the absence or presence of TMZ (Fig. 26A-B). We found that the radioactivity associated to intracellular S1P was similar in treated and untreated cells, meanwhile, intriguingly, extracellular [^{3}H]-S1P levels increased by 40% in cells exposed to TMZ with respect to untreated cells (Fig. 26C).

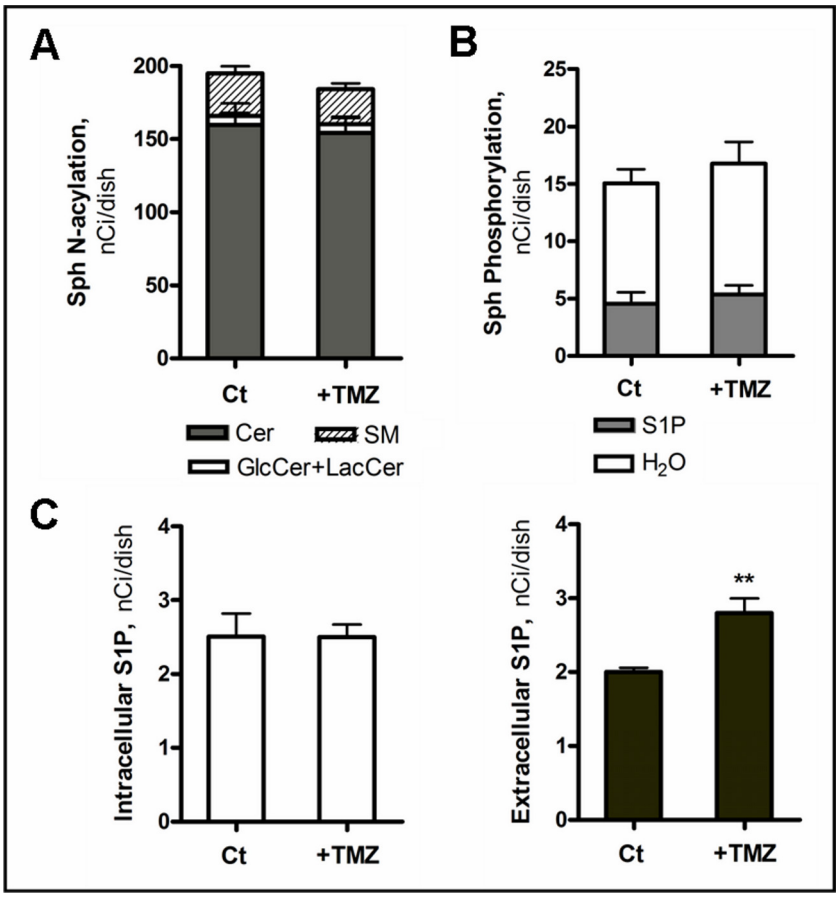

Figure 26. [^{3}H]-Sph metabolism in L0627 treated with TMZ. L0627 were treated with vehicle (Ct, 0.1% DMSO) or 100 μM TMZ (+TMZ) for 24 hours and then pulsed with 20 nM [^{3}H]-Sph (0.4 μCi/ml) for 60 minutes. At the end of incubation, cells and media were processed and analyzed as described in "Materials and methods". Radioactivity associated to the processes of sphingosine N-acylation **(A)** and sphingosine phosphorylation **(B)** are shown. **(C)** Radioactivity incorporated

into intracellular and extracellular S1P is represented. All data are expressed as nCi/dish. All values are mean ± SD of at least three independent experiments. Asterisks represent statistical significance determined by Student's t test (** $p < 0.01$ vs vehicle-treated cells).

5.13 Role of S1P in TMZ-induced toxicity in U87-MG

As reported above, U87-MG were sensitive to the cytotoxic action of 50-200 μM TMZ, whereas GSCs were resistant to these drug concentrations. Furthermore, U87-MG cells were characterized by markedly lower levels of extracellular S1P with respect to those of GSCs. We then examined whether exogenous S1P administration might be able to modulate U87-MG cell death by counteracting the cytotoxic effect of TMZ. To this purpose we administered to serum-starved cells 50 μM TMZ in the absence or presence of extracellular S1P (200 nM) for 48 hours.

The results obtained through MTT assay (Fig. 27), revealed that exogenously administered S1P by itself increased the number of viable cells by 20% with respect to control cells treated only with vehicle. Furthermore, the treatment with TMZ alone caused a 50% decrease in cell viability. Interestingly, exogenous S1P was able to reduce the cytotoxic effect of TMZ, determining a 2-fold increase in viable cell number, compared to cells treated only with TMZ.

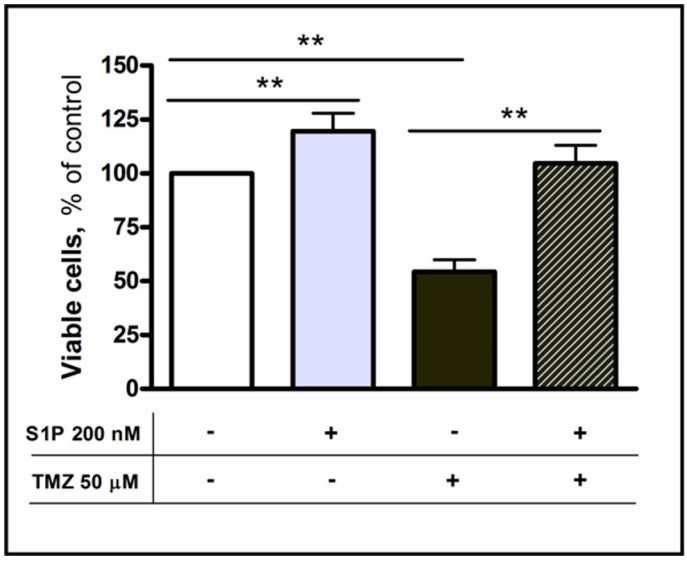

Figure 27. Effect of exogenous S1P on TMZ-induced toxicity in U87-MG. Cells were serum-starved for 24 hours and then incubated in serum-free medium containing 50 µM TMZ in the absence or presence of 200 nM S1P. After 24 hours 200 nM S1P was added again. Cell viability was assessed by MTT assay after 48 hours of treatment. Results are expressed as percentage of cell viability with respect to vehicle-treated cells (100%). Data are mean ± SEM of three independent experiments. Asterisks represent statistical significance determined by Student's t test (** $p < 0.01$).

5.14 Role of S1P in GSCs survival properties

Stimulated by these results, we investigated the putative role of S1P in GSCs resistance to TMZ. First of all, U-SC and L0627 were treated with different concentrations of TMZ (100-200 μM) in the absence or presence of the sphingosine kinase inhibitor SKI. After a 48 hours treatment, high doses of TMZ failed to induce cell death in U-SC and had only limited effects on L0627 survival. Moreover, at the administered concentration, the treatment with SKI alone did not significantly affect cell viability (data not shown). On the other hand, intriguingly, the co-treatment with SKI and TMZ dramatically decreased cell viability (Fig. 28). In particular, in U-SC treated with 100 μM TMZ in the presence of SKI, cell viability was decreased by 70%, compared to cells treated only with TMZ (Fig. 28A). Moreover, in L0627 the same treatment induced a decrease in cell survival of about 90% with respect to TMZ alone (Fig. 28B). Taken together these data suggest that SKI makes GSCs sensible to TMZ toxicity.

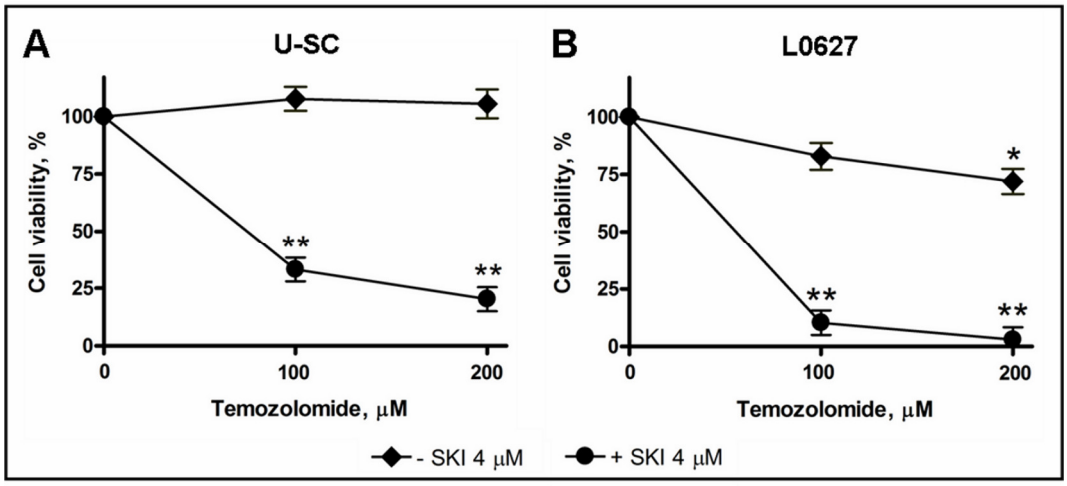

Figure 28. Role of S1P in GSCs survival properties. **(A)** U-SC and **(B)** L0627 were exposed to different doses of TMZ (100-200 μM) alone (♦) or in combination with 4 μM SKI (●). Cell viability was assessed by MTT assay after 48 hours of treatment. Results are expressed as percentage of cell viability with respect to vehicle-treated cells (♦) or cells treated with SKI alone (●) (100%). Data are mean ± SEM of at least three independent experiments. Asterisks represent statistical significance determined by Student's t test (* $p < 0.05$ and ** $p < 0.01$ vs vehicle-treated cells).

Subsequently, in order to assess the importance of extracellular S1P in GSC TMZ resistance, we evaluated the effect of S1P on L0627 in the presence or not of TMZ and SKI, separately or in combination. As shown in Figure 29, in control cells treated only with vehicle, the administration of exogenous S1P for 48 hours by itself slightly increased the number of viable cells. Moreover, in the presence of TMZ, S1P was able to promote a 40% increase in cell survival, thus indicating that S1P enhances GSC refractoriness to TMZ. Remarkably, exogenous S1P was able to strongly revert the sensitizing effect of SKI on TMZ toxicity determining a 5-fold increase in viable cells.

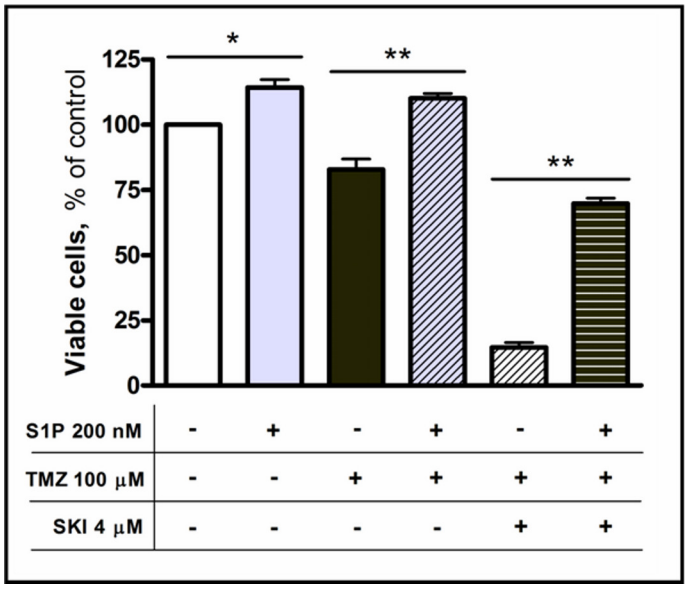

Figure 29. Extracellular S1P promotes GSCs resistance to TMZ. L0627 were exposed to 200 nM S1P, 100 µM TMZ, 4 µM SKI, separately or in combination, as indicated. After 24 hours 200 nM S1P was added again. Cell viability was measured by MTT assay after 48 hours of treatment. Results are expressed as percentage of viable cells with respect to vehicle-treated cells (100%). Data are mean ± SEM of at least three independent experiments. Asterisks represent statistical significance determined by Student's t test (* $p < 0.05$ and ** $p < 0.01$).

5.15 S1P production in GSCs isolated from two patients with a different GBM aggressive phenotype

The GSC ability to release S1P in the extracellular microenvironment stimulated us to evaluate its possible involvement in determining GBM aggressiveness. To this purpose, we used GSCs isolated from two GBM specimens obtained from patients showing a different prognosis. One patient (PT 1) was characterized by the absence of mutations in p53 oncosoppressor gene; meanwhile the other patient (PT 2) presented an inactivating mutation of p53. Interestingly, tumour in PT 2 had a higher grade of malignancy, showing a more aggressive phenotype, with respect to PT 1. Indeed only PT 2 death was consecutive to the formation of an early relapse.

Both GSC models were characterized by the high expression levels of the recognized cancer stem cell markers, CD133 and CD15 (Tab. 2).

	$CD133^{pos}$ cells (%)	$CD15^{pos}$ cells (%)
PT 1	47.3 ± 10.2	18.6 ± 2.5
PT 2	71.8 ± 15.3	10.2 ± 1.4

Table 2. Characterization of GSCs derived from two patients with a different GBM aggressive phenotype. CD133 and CD15 expression was assessed by flow cytometry analyses.

We evaluated S1P production in these two GSC models pulsing cells with tritiated sphingosine for 15, 30 minutes and 2 hours. Total incorporated radioactivity increased in a time-dependent manner and was similar in the two GSC models (data not shown). We found that the radioactivity associated with intracellular S1P remained constant in cells derived from PT 2, meanwhile it doubled at 30 minutes and remained constant at two hours of pulse in cells derived from PT 1 (Fig. 30A). However, intracellular [^{3}H]-S1P levels were similar in the two GSCs. Furthermore, in both cases the radioactivity associated with extracellular S1P increased with pulse time, tripling from 30 minutes to 2 hours. Intriguingly, we found that at all the investigated times extracellular [^{3}H]-S1P level was at least 4 times higher in GSCs derived from PT 2 with respect to those derived from PT 1 (Fig. 30B). These findings led to an extracellular/intracellular S1P ratio at least 3 times higher in GSCs from PT 2 than in those from PT 1 (Fig. 30C).

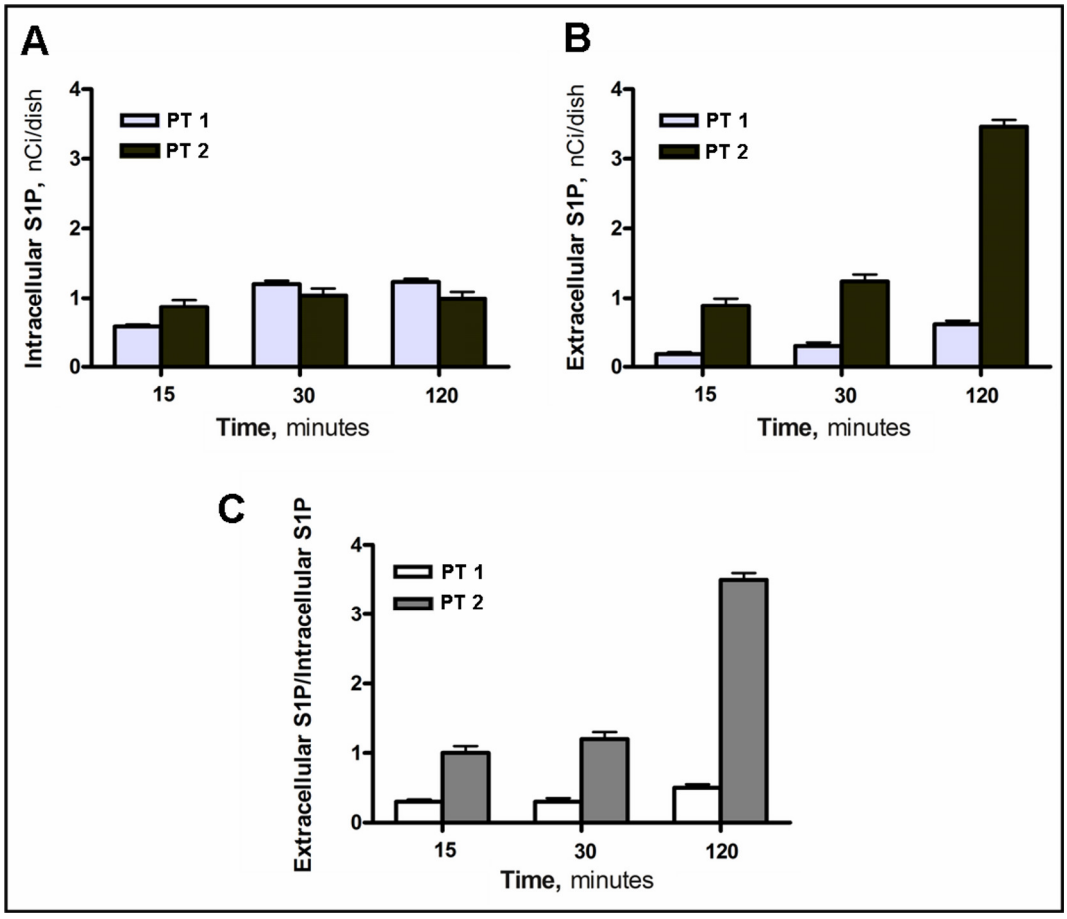

Figure 30. Intracellular and extracellular [^{3}H]-S1P in GSCs isolated from two patients with a different GBM aggressive phenotype. GSCs isolated from PT 1 and PT 2 were pulsed with 20 nM [^{3}H]-Sph (0.4 µCi/ml) for the indicated periods of time. At the end of incubation, cells and media were processed and analyzed as described in "Materials and methods". The radioactivity incorporated into intracellular S1P **(A)**, extracellular S1P **(B)** and the ratio between extracellular and intracellular S1P-associated radioactivity **(C)** are shown. All values are the mean ± SD of at least three independent experiments.

DISCUSSION

Glioblastoma multiforme is the most frequent and aggressive primary central nervous system tumour in humans, with one of the worst survival rates of all the human cancers [1], due to the high proliferation rate, the migrating and invasive properties, and, not last, the resistance to current therapeutic intervention. The introduction of the alkylating agent TMZ in glioblastoma therapy improved patient survival, but drug resistance mechanisms limit its benefits and the prognosis remains unfavorable [33]. The development of effective chemotherapy for gliomas is limited by a relative lack of understanding of the mechanisms of TMZ toxicity and chemoresistance in the tumour cells. Our study provides a contribution to this complex field.

First, to examine the antitumour effect of TMZ on malignant glioma cells, we used the human T98G glioblastoma multiforme cell line as cell model. Furthermore, since the establishment of cell lines with induced drug resistance is an effective model to clarfy the molecular basis of anticancer drug resistance, TMZ-R cells from T98G cells were generated and used. Both cell models were treated with increasing concentrations of TMZ. Our results demonstrated that TMZ, at doses that were cytotoxic in T98G cells, had no significant effect on TMZ-R viability, the resistance factor being five-fold greater than that of the parental T98G. Therefore, the expression of the DNA repair protein MGMT, a major contributor to TMZ-resistance [36] was assessed. We obtained that TMZ-R cells showed a marked increase in MGMT expression levels in comparison to T98G cells, indicating that MGMT has a role in TMZ-resistance in this cell line [252].

Accumulating literature indicates that ceramide plays an important role as a tumour suppressor lipid, able to induce antiproliferative and apoptotic responses, and that it is a major player in the mechanism of action of many chemotherapeutic drugs [107,260,261]. Therefore, we examined whether ceramide production is involved in the mechanism of TMZ-induced toxicity. Our data demonstrated that cytotoxic doses of TMZ induce a significant increase in ceramide in T98G glioma cells. Of note, this increase occurs prior to significant evidence of cell death (i.e., variation in total viable cells), suggesting a role of ceramide as a mediator of cytotoxicity. Interestingly, dead cells floating in the medium after TMZ treatment, which increased with treatment time, exhibited relevant higher levels of ceramide than those in vital, attached cells. Thus, it appears that TMZ increases ceramide, and when this increase reaches a certain level, cells undergo death. Furthermore, we found that in TMZ-R cells the administration of high doses of TMZ (highly cytotoxic in sensitive cells) failed to induce variations in ceramide content, as it occurs in T98G, supporting that this drug is not able to induce ceramide accumulation in resistant cells [252].

Prompted by these results, we next investigated ceramide effect on T98G viability. Our experiments demonstrated that ceramide analogs and ceramide itself were able to exert a cytotoxic effect in a dose-dependent manner.

From this first set of data, it emerges that TMZ affects ceramide formation, enhancing its production in T98G glioma cells, and that ceramide by itself appears to act as pro-death mediator of the drug effects [252].

Parallel studies in our laboratory demonstrated that both T98G and TMZ-R cells can release extracellularly newly synthesized S1P, the prosurvival antagonist of ceramide, and that S1P secreted by TMZ-R cells was significantly higher than that released by T98G cells. These findings suggest that S1P secretion in TMZ-R cells is functional to oppose the cytotoxic effect of ceramide and to the TMZ-resistant properties of cells.

Stimulated by these initial results, we next evaluated the role of sphingolipid mediators in the malignant features of GSCs, the cell subpopulation within the tumour involved in the aberrant expansion and therapy resistance properties of glioblastomas [50,51].

To this purpose we first used GSCs isolated from the human U87-MG glioblastoma multiforme cell line, known to be particularly enriched with cancer stem cells (U-SC) [263] and GSCs isolated from a primary culture of human glioblastoma (L0627). Both cellular models efficiently formed typical neurosphere structures in mitogen-defined medium and expressed high levels of recognized cancer stem cell markers CD133, nestin and CD15.

We found that TMZ, at doses that were cytotoxic in U87-MG cells, had no significant or only limited effect on GSCs viability. Of relevance, both GSCs were resistant to TMZ concentrations (100-200 μM) higher than that used *in vivo* in glioblastoma chemotherapeutic treatments. In fact, it was reported that levels of this drug in plasma and cerebrospinal fluid of patients range from 0.5 to 70 μM and from 0.8 to 10 μM, respectively [267]. These data, in agreement with previous studies in literature [50,51], indicate that both GSC models are resistant to TMZ, thus supporting that GSCs play a pivotal role in glioblastomas therapy resistance properties and malignancy.

Notwithstanding, the expression of the DNA repair protein MGMT, a major factor responsible for TMZ resistance [36], was undetectable not only in U87-MG, but surprisingly also in GSCs. This finding strongly suggests that GSCs resistance to TMZ is due to mechanisms different from an increased MGMT expression.

A large amount of evidence underlines the role of S1P as an important tumour-promoting lipid, favouring growth, invasion, and chemotherapy resistance of different cancer cells [247], including glioblastoma ones [235,248]. So far, little is known on the possible role of S1P as a factor modulating GSCs malignant properties. In the present study we provide evidence that directly supports GSCs as an important S1P

source in the extracellular microenvironment, which acts as an autocrine/paracrine signal contributing to the survival properties of GSCs.

In particular, we first performed immunoblotting analyses, which demonstrated that U87-MG and the two GSCs models express both the known isoforms of sphingosine kinase (SK1 and SK2), the enzyme responsible for S1P biosynthesis.

In addition, in order to evaluate the efficiency of S1P production and its metabolic fate, short time pulse experiments were performed in U87-MG and GSCs by administering to cells tritiated sphingosine as S1P precursor. The results of these experiments revealed the presence of radiolabeled S1P not only inside the cells, but also in the culture medium from both GSCs and U87-MG.

Notably the intracellular [^{3}H]-S1P levels were found much lower in GSC models than in U87-MG. On the other hand the extracellular [^{3}H]-S1P levels in GSCs were significantly higher than in U87-MG. These differences resulted in an extracellular/intracellular S1P ratio at least 10 times higher in GSCs compared to U87-MG. Furthermore, this ratio was about 1:1 in both GSCs, thus suggesting that these cells are an efficient source of S1P in the extracellular microenvironment.

Importantly, the S1P detection in GSCs medium was not accompanied by LDH release, demonstrating that the presence of S1P extracellularly was not a consequence of cell membrane rupture.

The differences in the extracellular S1P levels between U87-MG and GSCs resulted also in a ceramide-extracellular S1P ratio at least about 2-fold lower in GSCs than in U87-MG. Since S1P and ceramide exert opposite effects on cell survival, according to the “sphingolipid rheostat” model [111], this different ratio could promote GSC survival observed after TMZ treatment.

We then hypothesized that the relevant amount of S1P in GSCs medium could be consequent to SKs secretion in the extracellular milieu, as it occurs in some cell types [167-169]. However enzyme activity assays led us to exclude the presence of SKs in the GSC medium, suggesting that extracellular S1P derives from the export of newly synthesized intracellular S1P and that protein-mediated transport mechanisms are most probably involved in the S1P export from GSCs.

In this context, we analyzed in U87-MG and both GSCs the expression of ABCG2, ABCA1 and ABCC1, the members of the ABC-transporters family known to be involved in S1P export [173]. None of the analyzed transporters appeared to be expressed in GSCs, except for ABCA1. Data obtained by inhibiting this transporter in GSCs showed no significant variations in S1P release, thus leading us to exclude ABCA1 involvement in S1P export. In future studies it will be interesting to characterize the precise transport mechanism responsible for the extracellular release of S1P by GSCs.

A further interesting finding of our study is that GSCs treated with TMZ and then pulsed with tritiated sphingosine show a 40% increase in extracellular [^{3}H]-S1P levels in comparison with their untreated counterparts. On the other hand, no ceramide variations were observed after TMZ treatment, in agreement with the data obtained in T98G cells resistant to TMZ. This evidence leads us to hypothesize that GSCs are able to raise their extracellular S1P levels to protect themselves from the cytotoxic effect of TMZ regardless ceramide levels variations.

Since U87-MG were sensitive to TMZ and characterized by markedly lower levels of extracellular S1P with respect to GSCs, we then analyzed the putative S1P role in TMZ-induced toxicity in U87-MG cells. We found that exogenously administered S1P was able to significantly prevent TMZ-induced cell death, determining a 2-fold increase in viable cell number, compared to cells treated only with TMZ. These results demonstrated that exogenous S1P protects U87-MG cells against TMZ cytotoxic effect, contributing to their survival.

Stimulated by these findings and by the efficient ability of GSCs to product extracellular S1P, we then investigated the putative role of S1P in GSCs resistance to TMZ. First we demonstrated that the inhibition of S1P synthesis, through a SK inhibitor, made both GSC models sensitive to TMZ toxicity. Furthermore exogenous S1P reverted the cytotoxic effect of the concomitant treatment with TMZ and SK inhibitor. These data strongly support extracellular S1P as an important mediator in TMZ-resistance of GSCs.

In parallel, in order to better clarify S1P role in determining GBM aggressiveness from a clinical point of view, we analyzed S1P production in GSCs isolated from two patients affected by GBM with different aggressive phenotype and prognosis. Our preliminary data show that GSCs isolated from the patient with the most aggressive GBM were able to release an amount of S1P at least 4 times higher than those from the other patient. These results suggest that GSC extracellular S1P could have a peculiar role in determining GBM aggressiveness and patient prognosis. It will be interesting to evaluate extracellular S1P contribution in a higher number of GBM patients, in order to better verify our hypothesis.

Altogether these data implicate, for the first time, GSCs as an important S1P source in the extracellular microenvironment, where, on its turn, S1P can act as an autocrine/paracrine messenger able to contribute to the GSCs aggressiveness and survival properties. In particular, the presence of S1P in the extracellular environment promotes GSC survival and resistance after TMZ treatment. Furthermore, the inhibition of S1P synthesis, and thus the reduction of its release, makes cells sensitive to this chemotherapic agent.

Moreover, our findings provide a possible rationale for the results of a very recent study on FTY720, a sphingosine analogue that, once phosphorylated by SKs, initially activates S1P receptors via high-affinity

binding, inducing subsequently their down-regulation [246]. Estrada-Bernal and colleagues in this study demonstrated that the *in vitro* administration of FTY720 decreases GSCs viability and acts synergistically with TMZ. Moreover, *in vivo* FTY720 promotes survival in a rodent model of GBM and decreases GSCs invasiveness in nude mouse brains. Taken together these and our results let us to speculate that S1P is efficiently produced within GSCs and, once released, acts through its receptors determining drug-resistance and promoting growth and invasion of GSCs, thus contributing to glioblastoma malignancy.

A better understanding of S1P role in determining GSC malignant properties and its mechanism of action could help to identify new targets for the development of new therapies that may be associated with chemotherapy treatments already in use. Therefore, this study not only sets the basis for a greater comprehension of the mechanisms regulating GSC behavior, but also for the future development of efficient and effective therapies against GBM such as new compounds able to modulate S1P metabolism to strongly sensitize GSCs to chemotherapeutic treatments, thus improving survival rates and ensuring a long-term life perspective in GBM patients.

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